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Retinoids, the natural and synthetic vitamin A derivatives, are well known for their inhibitory effect on the proliferation of breast cancer cells. However, the growth inhibitory effect of retinoids appears to diminish during progression of breast tumor as the effect is mainly observed in hormone-dependent estrogen receptor (ER) positive breast cancer cells but not in hormone-independent ER negative breast cancer cells. The goal of this research project is to investigate the molecular mechanisms by which retinoids exert their differential growth inhibitory effects on hormone-dependent and -independent breast cancer cells. Our data demonstrate that retinoids can antagonize the mitogenic effect of estrogen through their negative regulation of ER transactivation activity on estrogen response elements. In addition, a loss of retinoic acid receptor β may be responsible for retinoid resistance of hormone-independent breast cancer cells. Furthermore, our data demonstrate that induction of apoptosis and anti-AP-1 activity contribute to retinoid-induced growth inhibition. These results largely enhance our understanding of the mechanism of retinoid action in breast cancer cells and also point to a possibility of restoring retinoid sensitivity in hormone-independent breast cancer cells.

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INTRODUCTION

Retinoids, the natural and synthetic vitamin A derivatives, are currently used in the treatment of epithelial cancer and promyelocytic leukemia and are being evaluated as preventive and therapeutic agents for a variety of other human cancers. They have shown promise as new preventive as well as therapeutic agents for breast cancer in various model systems. In animals, administration of retinoids inhibits the initiation and promotion of mammary tumors induced by carcinogens. In vitro, retinoids were shown to inhibit the growth of human breast cancer cells, majority of which, however, are hormone-dependent estrogen receptor (ER) positive breast cancer cells. Inhibition on growth of such cells in culture has been observed when retinoids are administered alone or in combination with anti-estrogen, where synergistic effects are observed. Therefore, retinoids are under extensive clinical trials. However, early clinical trials of retinoids on patients with advanced breast cancer have not demonstrated significant activities. This may be due in part to the loss of retinoid sensitivity in hormone-independent breast cancer cells and suggests the possible modification of retinoid response during progression of breast tumor. Further evaluation of retinoids for breast cancer treatment, therefore, is largely dependent on a better understanding of the molecular mechanism by which they act as anti-breast cancer agents.

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). RARs and RXRs are part of the steroid/thyroid hormone receptor superfamily. Both types of retinoid receptors are encoded by three distinct genes, α, β and γ. These receptors, through heterodimerization and homodimerization, function as ligand-activated transcription factors that bind to specific responsive sequences on the target genes and regulate the transcriptional expression of these genes. One of the target genes is RARβ itself and a RA response elements (βRARE) has been identified in its promoter region, that mediates the auto-induction of RARβ gene expression. Alteration of retinoid receptor activity could be associated with neoplastic transformation as demonstrated by abnormal RARα transcripts found in patients with acute promyelocytic leukemia (APL).

Despite extensive research on retinoid response in breast cancer cells, how the inhibitory effect of retinoids on breast cancer cell growth is mediated and how this inhibitory effect is lost in hormone-independent ER negative breast cancer cells are large unclear. Since the effects of retinoids are mainly mediated by their nuclear receptors, several studies were initiated to examine the expression of RAR genes in a number of human breast cancer cell lines and showed RAR genes are expressed in the breast cancer cell lines examined. Although variations in the expression levels of certain RAR genes were observed, such minor variations could not explain the dramatic difference in the response of these breast cancer cell lines to retinoids and a more detailed analysis of the involvement of retinoid receptors is needed. Since the growth inhibitory effects of retinoids appear to be ER status dependent, it is also likely that ER functions as a target of retinoid action. We propose that the anti-estrogen effect of retinoids may be one of the major mechanisms by which they act to inhibit breast cancer cell growth and that the loss of retinoid sensitivity during progression of breast tumor may be due to alterations of retinoid receptor activities. The purpose of this research is to investigate the molecular mechanisms by which retinoids exert their growth inhibitory effects on breast cancer cells and how the effects are lost in hormone-independent ER negative breast cancer cells. The results from these studies may provide a molecular basis of developing more effective preventive and therapeutic retinoids and treatment strategies against breast cancer.

BODY

Interaction between retinoid and estrogen signalings (Lee at al., 1995). We have recently identified a retinoic acid response element (RARE) in the 5'-flanking region of the lactoferrin gene promoter. The lactoferrin-RARE is composed of two AGGTCA-like motifs arranged as a direct repeat with one base pair spacing (DR-1). Gel retardation assay demonstrated that it bound strongly with retinoid X receptor (RXR) homodimers and RXR/retinoic acid receptor (RAR) heterodimers as well as COUP-TF orphan receptor. In CV-1 cells, the lactoferrin-RARE linked with a heterologous thymidine kinase promoter was strongly activated by RXR homodimers in response to 9-cis RA but not to all-trans RA. When coup-TF orphan receptor was cotransfected, the 9-cis RA induced RXR homodimer activity was strongly repressed. An unique feature of the lactoferrin-RARE is that it shares an AGGTCA-like motif with an estrogen responsive element (ERE). The composite

RARE/ERE contributes to the functional interaction between retinoid receptors and ER and their ligands. In CV-1 cells, cotransfection of the retinoid and estrogen receptors led to a mutual inhibition of each other's activity while a RA-dependent inhibition of ER activity was observed in breast cancer cells. Furthermore, the lactoferrin-RARE/ERE showed differential transactivation activity in different cell types. RAs could activate the lactoferrin-RARE/ERE in human leukemia HL-60 cells and U937 cells but not in human breast cancer cells. By gel retardation analyses, we demonstrated that strong binding of the endogenous COUP-TF in breast cancer cells to the composite element contributed to diminished RA response in these cells. Thus, the lactoferrin RARE/ERE functions as a signaling switch modular that mediates multihormonal responsiveness in the regulation of lactoferrin gene expression. Importantly, this study demonstrates a novel mechanism by which retinoid and estrogen signalings interact. We have further extended this observation and shown that inhibition of estrogen-induced ER activity can be observed even on a putative ERE in various breast cancer cells. These results, therefore, suggest that inhibition of ER activity may represent an important mechanism by which retinoids inhibit the growth of hormone-dependent ER positive breast cancer cells.

Binding of a RXR containing complex on the ERE. To study the mechanism by which RA inhibits estrogen-induced ER transactivation activity, we investigated the possibility that a nuclear protein present in the breast cancer cells might interact with RXR and form an ERE binding complex. RXR alone did not exhibit any DNA binding on ERE (fig. 1). However, when it was mixed with nuclear proteins prepared from hormone-dependent ZR-75-1 cells, we observed a prominent new DNA binding complex (fig 1). Interestingly, this new complex was not found when nuclear proteins from hormone-independent MB468 or CV-1 cells were used (fig. 1). Thus, competition for ERE binding by the complex formed with RXR and a nuclear protein present in hormone-dependent breast cancer cells may be one of the mechanisms for retinoids to antagonize ER activity. This result suggests that a cell type-specific nuclear protein present in breast cancer cells may mediate the anti-estrogen effect of retinoids.

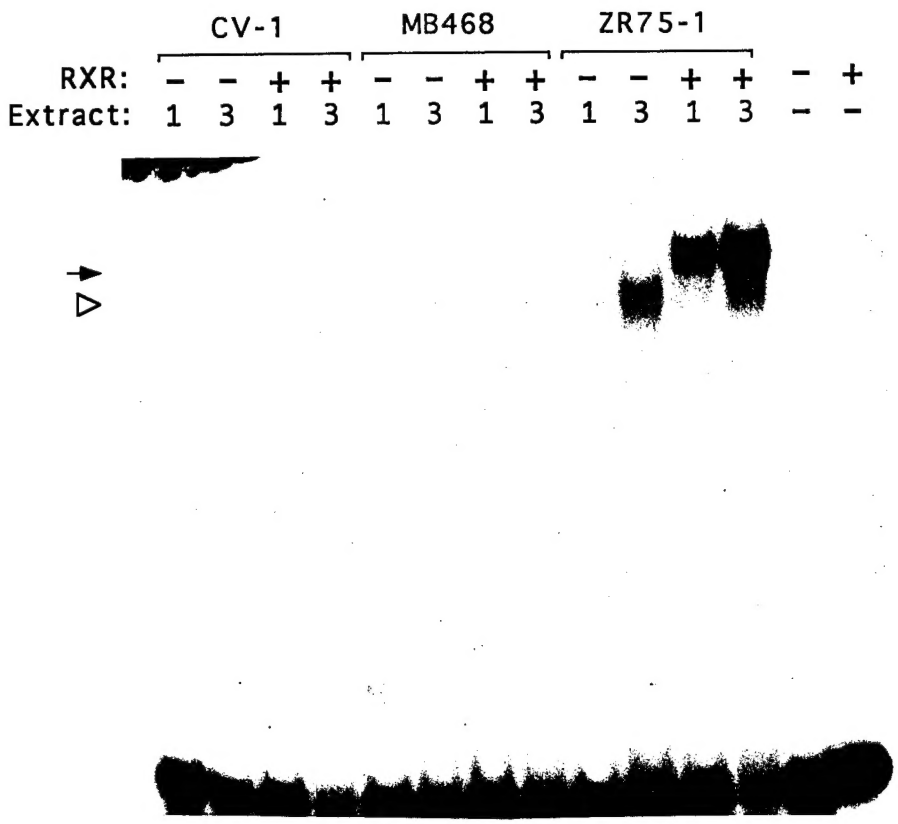

Figure 1. Interaction of RXR with a nuclear protein from human breast cancer cells. RXRα protein was incubated with or without indicate amount (ng) nuclear extract prepared from CV-1, MB468 or ZR-75-1 cells. After incubation for 15 min at room temperature, the mixtures were analyzed by gel retardation assay using consensus ERE as a probe. Open triangle indicates the binding of nuclear protein prepared from ZR-75-1 cells. Arrow indicates the binding complex observed when RXR was mixed with nuclear extract.

RARβ mediates the growth inhibitory effect of retinoids in breast cancer cells (Liu et al., 1995). Retinoids are known to inhibit the growth of hormone-dependent but not of hormone-independent breast cancer cells. To establish the involvement of retinoid receptors in the differential growth inhibitory effect of RA on breast cancer cells, we examined the expression of three types of RAR (α, β, and γ) and of RXR (α, β and γ) in various hormone-dependent and -independent breast cancer cell lines. Transcripts for RARα, RARγ, RXRα, and RXRβ were detected in all the cell lines with minor variations in expression levels while

the expression of RXRγ gene was not observed under the conditions used. When the expression of the RARβ gene was examined, we found that it was strongly enhanced by RA in hormone-dependent breast cancer cell lines while treatment with RA failed to induce its expression in hormone-independent cell lines. In examining the growth inhibitory effect of all-trans RA and 9-cis RA, we observed that both RAs showed a strong inhibition of the growth of hormone-dependent cell lines while they had no effect on hormone-independent lines. Thus, the induction of RARβ gene expression by RA correlates to the growth inhibitory effect of RAs, suggesting that RARβ may mediate the RA effect. To directly test this, cDNA for the RARβ gene was stably expressed in hormone-independent breast cancer cells (MB231). When the anchorage-dependent and -independent growth of RARβ transfectants was measured in the presence and absence of RA, we observed a strong growth inhibition of RARβ transfectant cells in the presence of RA. Thus, the expression of the RARβ gene can restore the growth inhibitory effect of RA in hormone-independent breast cancer cells. In addition, RA sensitivity of hormone-dependent cells was inhibited by a RARβ-selective antagonist and the expression of RARβ anti-sense RNA. Together, RARβ can mediate retinoid action in breast cancer cells and the loss of RARβ may contribute to the tumorigenicity of human mammary epithelial cells.

RA promotes apoptosis in breast cancer cells (Liu et al., 1995). Apoptosis or programmed cell death is a fundamental important physiologic process in normal development and tissue homeostasis. Although the biochemical pathway that mediates apoptosis is still unknown, in many cases apoptosis is controlled by a number of factors including extracellular ligands such as steroids, growth factors, intracellular mediators of signal transduction, nuclear proteins regulating gene expression, DNA replication, and cell cycle. Recently, apoptosis has been recognized as another important pathway that helps restrict cell proliferation. Suppression of normal apoptosis can result in abnormal cell survival and malignant growth. Several studies have demonstrated that tumor cells can be eliminated by artificially triggering death through apoptosis. When RARβ was expressed in MB-231 cells, we noticed that many RA-treated cells detached and became shrunken, followed by cell death. When the nuclei of these cells were stained by propidium iodine (PI) and examined by fluorescence microscopy, we found that many of the RA-treated cells were smaller and contained fragmented nuclei with brightly staining chromatin, i.e., morphological changes typical of apoptosis. RA caused similar morphological changes in RA-sensitive ZR-75-1, MCF-7 and T-47D cells but not in the RA-resistant MB-231 and MB-468 cells. Furthermore, DNA fragmentation indicative of apoptosis was also detected in ZR-75-1 and MB-231 introduced with RARβ.

DNA fragmentation was found in more than half of the RA-treated ZR-75-1 cells, and occurred as early as 12 hours after exposing cells to RA. Interestingly, ZR-75-1 cells that expressed RARβ anti-sense RNA experienced significantly less DNA fragmentation in response to RA. Thus, RA can induce apoptosis in RA-sensitive hormone-dependent breast cancer cells, which is likely mediated by RARβ. Together, retinoids may suppress breast cancer cell growth by the process of apoptosis.

RA inhibits TPA-induced AP-1 activity in breast cancer cells. The mechanism by which retinoids exert their anti-cancer activity is largely unknown. Since cancer is a malignancy in which the balance between growth and differentiation, as well as cell renewal and cell loss is disturbed, the anti-cancer activity of retinoids is believed to be at least partially due to their direct anti-proliferative effects. This has been observed in a series of transformed cell lines, including mammary, melanoma, lymphoid and fibroblastic. The mitogenic stimuli, often generated by the autocrine secretion of growth factors is transmitted to the cell nucleus via certain second messenger pathways. Although starting out from different growth factors and the usage of distinct pathways, it is often the activation of the nuclear transcriptional factors cJun and cFos, the component of AP-1, that trigger cell proliferation. We have previously shown that RARs, in response to RA, can antagonize the activities of c-jun and c-fos. Such interactions between membrane and receptor pathways in nucleus may be essential for the control of cellular proliferation. To determine whether breast cancer cells contain TPA-induced AP-1 activities, -73Col-CAT, that contains collagenase promoter linked with CAT gene, was transfected into ZR-75-1 or T-47D cells. When cells were treated with TPA, we observed a strong induction of reporter activity, which was significantly inhibited when RA was present (fig. 2). These results suggest that under certain conditions the anti-AP-1 activity of retinoids may contribute to the growth inhibitory effect of *trans*-RA in these breast cancer cells.

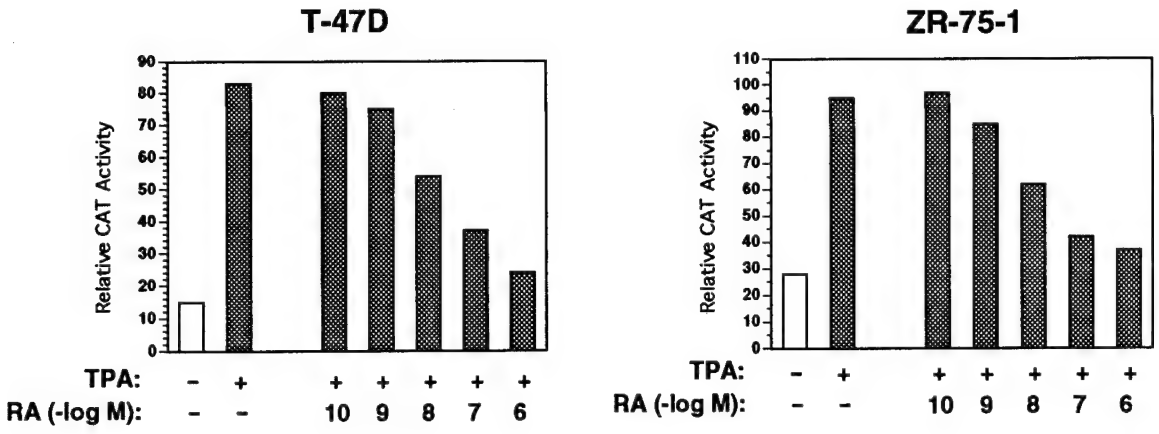

Figure 2. Inhibition of TPA-induced AP-1 activity in breast cancer cells by trans RA and retinoids. ZR-75-1 and T-47D cells were transiently transfected with 250 ng of -73ColCAT reporter gene. After transfection, cells were grown in 0.5% charcoal-treated FCS in the presence or absence of indicated amounts of *trans*-RA with or without TPA (100 ng/ml^{-1}) for 24 h. The β-gal activity was used to normalize for CAT activity. The assay were as described previously.

Conclusion.

Our studies have elucidated mechanisms by which retinoids differentially inhibit the growth of hormone-dependent and -independent breast cancer cells. The results obtained demonstrate that the loss of retinoid sensitivity in hormone-independent breast cancer cells is likely due to a loss of either the target of retinoid action (ER) or effector (RARβ) or both. Furthermore, we have shown that RA can induce apoptosis and inhibit AP-1 activity in breast cancer cells. Both mechanisms may contribute to the growth inhibition of breast cancer cells depending on cell type or growth conditions. The discovery that the loss of RARβ is responsible for the diminished RA sensitivity in hormone-independent breast cancer cells provides directions to restore retinoid sensitivity in certain malignant breast cancer cells and to develop more effective retinoids against breast cancer. As proposed we will investigate the molecular mechanism by which retinoid inhibit ER activity by determining the nature of

RXR-containing complex on ERE. Due to our exciting finding of RARβ, we will also further investigate the function of RARβ and the mechanism by which RARβ is abnormally expressed in hormone-independent breast cancer cells.

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A Retinoic Acid Response Element That Overlaps an Estrogen Response Element Mediates Multihormonal Sensitivity in Transcriptional Activation of the Lactoferrin Gene

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The lactoferrin gene is highly expressed in many different tissues, and its expression is controlled by different regulators. In this report, we have defined a retinoic acid response element (RARE) in the 5′-flanking region of the lactoferrin gene promoter. The lactoferrin-RARE is composed of two AGGTCA-like motifs arranged as a direct repeat with 1-bp spacing (DR-1). A gel retardation assay demonstrated that it bound strongly with retinoid X receptor (RXR) homodimers and RXR-retinoic acid receptor (RAR) heterodimers as well as chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan receptor. In CV-1 cells, the lactoferrin-RARE linked with a heterologous thymidine kinase promoter was strongly activated by RXR homodimers in response to 9-*cis*-retinoic acid (9-*cis*-RA) but not to all-*trans*-RA. When the COUP-TF orphan receptor was cotransfected, the 9-*cis*-RA-induced RXR homodimer activity was strongly repressed. A unique feature of the lactoferrin-RARE is that it has an AGGTCA-like motif in common with an estrogen-responsive element (ERE). The composite RARE/ERE contributes to the functional interaction between retinoid receptors and the estrogen receptor (ER) and their ligands. In CV-1 cells, cotransfection of the retinoid and estrogen receptors led to mutual inhibition of the other's activity, while an RA-dependent inhibition of ER activity was observed in breast cancer cells. Furthermore, the lactoferrin-RARE/ERE showed differential transactivation activity in different cell types. RAs could activate the lactoferrin-RARE/ERE in human leukemia HL-60 cells and U937 cells but not in human breast cancer cells. By gel retardation analyses, we demonstrated that strong binding of the endogenous COUP-TF in breast cancer cells to the composite element contributed to diminished RA response in these cells. Thus, the lactoferrin-RARE/ERE functions as a signaling switch module that mediates multihormonal responsiveness in the regulation of lactoferrin gene expression.

Retinoic acid (RA) and its natural and synthetic derivatives of vitamin A (retinoids) play a pivotal role in many aspects of vertebrate development and in the establishment and maintenance of physiological processes in adult tissues (40, 58). The effects of retinoids are mainly mediated by two classes of nuclear receptors, the retinoic acid receptors (RARs) (3, 5, 16, 17, 32, 54) and the retinoid X receptors (RXRs) (22, 36, 44, 45, 68). RARs and RXRs are part of the steroid-thyroid hormone receptor superfamily (14, 21) that also includes receptors for estrogen and vitamin D. These receptors function as ligand-activated transcriptional factors that bind to specific response sequences on the target genes and thereby regulate the transcriptional expression of these genes (14, 21). Both types of retinoid receptors are encoded by three distinct genes, α, β, and γ (3, 5, 16, 17, 22, 32, 36, 44, 45, 54, 68). All-*trans*-RA and 9-*cis*-RA (24, 37), the two known active derivatives of vitamin A, essentially function as hormones by interacting with specific retinoid receptors. For transactivation, RARs require interaction with RXRs, resulting in the formation of RAR-RXR heterodimers that recognize an RA response element (RARE), usually consisting of AGGTCA or like core motifs arranged as a direct repeat with either 2- or 5-bp spacing (9, 29, 32a, 34, 36, 43, 47, 50, 68, 69–72). RXRs can also heterodimerize with several other nuclear hormone receptors, including thyroid hormone receptors, vitamin D receptor, peroxisome proliferator-activated receptor, and most likely other nuclear proteins (see reference 71 for a review). In addition, RXR can function as homodimers (33, 35, 70–72), in the presence of its ligand, 9-*cis*-RA, that recognize DR-1 (direct repeat with 1-bp spacing)-type RAREs (32a, 35, 70, 72) or an inverted repeat element with 0-bp (70, 72) or 9-bp spacing (33). The magnitude of the RA response can also be affected by the expression of other transcriptional factors. Several studies (10, 28, 62, 67) have demonstrated that chicken ovalbumin upstream promoter transcription factor (COUP-TF) orphan receptors can restrict retinoid response, probably through their strong DNA binding to the RAREs or through dimerization with RXR.

The unusual homo- and heterodimerization capacity of RXR not only greatly expands the repertoire of regulatory diversity and specificity of nuclear hormone receptors but also allows RXR and its ligand, 9-*cis*-RA, to serve as important regulators in the interaction of retinoid and other signaling transduction pathways. Some of the dominant negative effects of v-*erbA* (12) and unliganded thyroid receptor (19, 73) on RA action could be achieved at the receptor level through their competition with RARs for heterodimer formation with RXR (23, 35). The formation of RXR homodimers can lead to another level of hormonal interaction induced by RXR ligand. For instance, 9-*cis*-RA can repress thyroid hormone (35) or vitamin D (42) activity through its ability to shift RXR from heterodimer to homodimer formation. The interaction of retinoid with other signal transduction pathways can also be achieved by the overlapping of RARE and other transcriptional factor-binding sequences. An overlapped RARE and AP-1 binding site, which mediates tetradecanoyl phorbol acetate responsiveness, has been demonstrated to mediate the interaction between nuclear hormone and cellular signal trans-

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duction pathways (59). Similarly, a single response element can function as an RARE and vitamin D response element, mediating the cross-talk between RA and vitamin D (59).

Lactoferrin is a nonheme iron-binding glycoprotein with multiple functions (48). It can inhibit the growth of bacteria by chelating iron (49) and contributes to the anemia of chronic disease (4). In addition, it was shown that lactoferrin can inhibit myelopoiesis (8). Recent studies (18, 25, 31, 38, 39, 53) have suggested that nuclear hormone receptors play a critical role in the regulation of lactoferrin gene expression. The high level of lactoferrin found in various mammary gland (48) and female reproductive organs (48, 53) led to identification of an estrogen response element (ERE) in the 5′-flanking region of the lactoferrin gene promoter (39). The ERE conferred estrogen-stimulated transcription in the presence of estrogen receptor (ER) (39), which could be repressed by COUP-TF because of its strong binding to the region (38). Lactoferrin is also highly expressed in hematopoietic cells at late stages of granulocytic differentiation (31, 48, 49, 56, 57), suggesting that expression of the lactoferrin gene in these cells is likely regulated very differently. However, little is known about how expression of the lactoferrin gene is regulated during the differentiation process of myeloid cells. RA and its receptors are well known to regulate the process of granulocytic differentiation as well as neutrophil maturation (6, 7, 31). During the terminal differentiation of 32D C13 cells, a process that is known to be regulated by RA (31), lactoferrin is highly expressed (18). In addition, treatment of human acute promyelocytic leukemia cells with RA led to an enhanced expression of the lactoferrin gene (25). These observations suggest that RA may be involved in the regulation of lactoferrin gene expression.

Here, we report the identification of a an RARE in the 5′-flanking region of the lactoferrin gene. The lactoferrin-RARE is composed of two AGGTCA-like motifs arranged as direct repeats with 1-bp spacing and is strongly activated by RXR homodimers in response to 9-*cis*-RA. Interestingly, one of the AGGTCA-like sequence of the lactoferrin-RARE is also used to form ERE. The overlapped hormone response element allowed functional interaction between the retinoid and estrogen receptors and their ligands. Furthermore, our results demonstrated that COUP-TF, which is differentially expressed in different cell types, dictated both RA and estrogen responses through its strong binding to the DR-1 part of the composite element. Thus, the lactoferrin composite hormone response element can mediate differential hormone sensitivities and may serve as a major signaling switch in the regulation of lactoferrin gene expression.

MATERIALS AND METHODS

Plasmid constructions. The reporter plasmids lactoferrin-RARE/ERE-tk-CAT and lactoferrin-RARE-tk-CAT were constructed by inserting three copies of the following synthetic sequence with additional 5′-GATC overhangs into the *Bam*HI site of pBLCAT$_2$ (41): the lactoferrin-RARE/ERE, AAGTGTCACAG GTCAAGGTAACCCAC, and the lactoferrin-RARE, AAGTGTCACAGGT CAAG. Mutated lactoferrin-RARE/ERE oligonucleotides were also synthesized and cloned into the *Bam*HI site of pBLCAT$_2$. They are lactoferrin-RARE/ERE-m1, AAGTGTCACAGGTCAAGGTATCCCA; lactoferrin-RARE/ERE-m2, AAGTGTCACAGGACAAGGTAACCCA; and lactoferrin-RARE/ERE-m3, AAGTGTACACAGGTCAAGGTAACCCA (the underlined letter indicates the changed nucleotide). The constructs were sequenced to verify the copy number and orientation (RARE/ERE, three copies, →→←; RARE, three copies, ←←→; m1, one copy, ←; m2, one copy, →; m3, one copy, →). The βRARE-tk-CAT reporter has been described previously (26). The coding sequences of RXRα, RARα, ER, ERval, and COUP-TF were inserted into the multiple cloning sites of the eukaryotic expression vector pECE or pBluescript. The construction of these plasmids has been described elsewhere (62, 64, 69).

Tissue culture, transient transfection, and CAT assay. The breast cancer cell lines ZR-75-1, Hs578T, and T-47D were obtained from the American Type Culture Collection. ZR-75-1, HL-60, and T-47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS); CV-1, U-937, and Hs578T cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% FCS. A modified calcium phosphate precipitation procedure was used for transient transfection and is described elsewhere (55). For CV-1 cells, the transfection assays were carried out in a 24-well culture plate. Reporter plasmid (100 ng), 150 ng of β-galactosidase (β-gal) expression vector (pCH110; Pharmacia), and variable amounts of receptor expression vector were mixed with carrier DNA (pBluescript) to 1 μg of total DNA per well. For ZR-75-1, Hs578T, HL-60, and U-937 cells, the transfection assays were carried out in six-well culture plates. Reporter (400 ng), 600 ng of β-gal, and variable amounts of receptor expression vectors were mixed with carrier DNA to 4 μg of total DNA per well. For HL-60 and U-937 cells, cells were treated with 10% glycerol after transfection. Chloramphenicol acetyltransferase (CAT) activity was determined by a modified assay as described before (55). Counts per minute, normalized for transfection efficiency by the corresponding β-gal activity, were expressed as relative CAT activity.

Preparation of nuclear extracts. Nuclear extracts were prepared essentially by the method of Andrew and Faller (2) with minor modification. Briefly, cells growing at about 90% confluency were washed with cold phosphate-buffered saline (PBS) and scraped into PBS with a rubber policeman. Cells were pelleted by low-speed centrifugation and then resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $CaCl_2$, and 2 mM $MgCl_2$. After pelleting, the cells were lysed in buffer containing 1% Nonidet P-40 by 10 to 15 strokes with an ice-cold Dounce homogenizer. Immediately after lysis, nuclei were collected by centrifugation at 2,000 × *g* and washed once with a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol (DTT). Nuclear proteins were extracted with a high-salt buffer containing 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT. All the buffers used for the procedure contained protease inhibitors, i.e., phenylmethylsulfonyl fluoride (PMSF), 100 μg/ml; leupeptin, 1 μg/ml; and aprotinin, 1 μg/ml. When it was necessary, nuclear extracts were concentrated by Centricon 10 (Millipore). Small aliquots of nuclear proteins were immediately frozen and kept at −80°C until use.

Preparation of receptor protein. The bacterially expressed RXRα and RARγ proteins have been described previously (69, 70). cDNA for the receptor was cloned in frame into the expression vector pGEX-2T (Pharmacia). The protein was expressed in bacteria following the conditions recommended by the manufacturer and purified on an affinity glutathione-Sepharose 4B column. To synthesize receptor protein in vitro, DNA sequences encoding ER or COUP-TF cloned into pBluescript were transcribed by using T7 or T3 RNA polymerase, and the transcripts were translated in the rabbit reticulocyte lysate system (Promega) as described before (69). The relative amounts of the translated proteins were determined by separating the [^{35}S]methionine-labeled proteins on sodium dodecyl sulfate-polyacrylamide gels, quantitating the amounts of incorporated radioactivity, and normalizing it relative to the content of methionine residues in each protein.

Gel retardation assays. Bacterially expressed or in vitro-synthesized receptor proteins or nuclear extract was incubated with ^{32}P-labeled oligonucleotides in a 20-μl reaction mixture containing 10 mM HEPES buffer (pH 7.9), 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI-dC) at 25°C for 20 min. The reaction mixture was then loaded on a 5% nondenaturing polyacrylamide gel containing 0.5× TBE (1× TBE is 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). When antibody was used, it (1 μl) was incubated with nuclear extract for 30 min at room temperature before performance of the DNA-binding assay. The polyclonal anti-RXR and anti-RAR antibodies were kindly provided by A. Lombardo (La Jolla Cancer Research Foundation, La Jolla, Calif.). They were raised in rabbits against the ligand-binding domain of RXR and RAR and showed cross-reactivity with different RXRs (α, β, and γ) and RARs (α, β, and γ). The oligonucleotides used for gel retardation assays were lactoferrin-RARE/ERE (AAGTGTCACAGGTCAAGGTAACCCAC), lactoferrin-RARE (AAGTGTCACAGGTCAAG), lactoferrin-ERE (CAGGT CAAGGTAACCCAC), DR-1 (GGGTCACGGGTCA), ERE (TCAGGTCAC TGTGACCTGA), GRE (CTCAGAACACAGTGTTCTGAGCATG), and SP-1 (CCCGAGGTGGGCGGGTGAGGCC). The oligonucleotides were labeled by Klenow DNA polymerase, and the labeled oligonucleotides were purified by gel electrophoresis and used as probes for the gel retardation assay.

RESULTS

Identification of the DR-1-type RARE that overlaps an ERE in the 5′-flanking region of the lactoferrin gene promoter. The expression of the lactoferrin gene is induced during the terminal differentiation of myeloid cells, in which RAs are well known as a potent regulator (6, 7, 31, 63). To investigate the possible involvement of RAs and their receptors in the regulation of lactoferrin gene expression, we used a CAT reporter gene (0.6 mL14-CAT [39]) that contains the 5′-flanking region

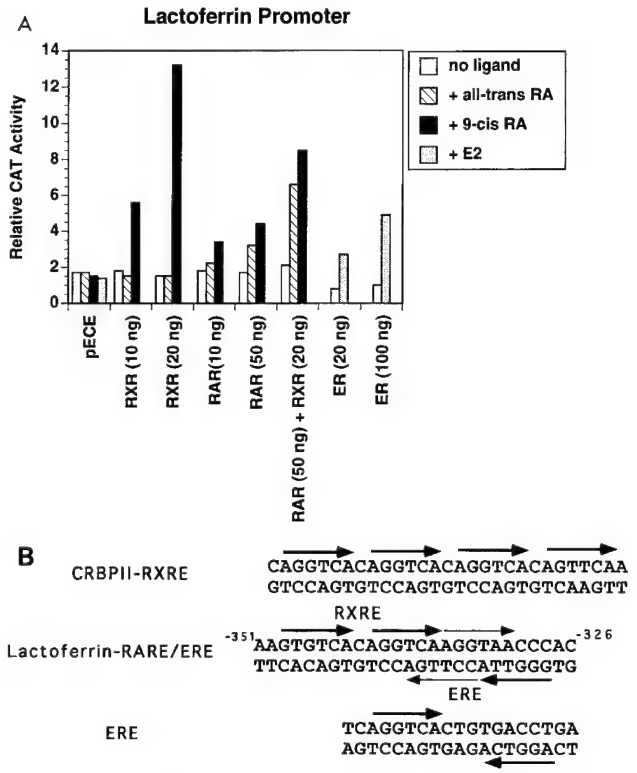

FIG. 1. Transcriptional activity of the lactoferrin gene promoter in CV-1 cells. (A) Regulation of the transcriptional activity of the lactoferrin gene promoter by RAs and their receptors. p0.6 mL14-CAT was cotransfected with empty vector (pECE) (20 ng) or the indicated amounts of RARα, RXRα or ER or RARα and RXRα together into CV-1 cells. Transfected cells were not treated or treated with ligand (all-*trans*-RA or 9-*cis*-RA, 10^{-7} M; E_2, 10^{-8} M) and assayed 24 h later for CAT activity. Data shown represent the means of two independent experiments. To ensure optimal detection of E_2-induced ER activity, the ERval expression vector (64) was used in this experiment. (B) Sequence comparison of the RARE in the lactoferrin gene promoter and the RARE from the CRBP-II gene promoter and a consensus ERE. Sequences that are closely related to the AGGTCA motif are indicated by thick arrows, whereas less related sequences are shown by thin arrows.

of the lactoferrin gene promoter (−589 to −21) to determine its response to RAs in CV-1 cells. As shown in Fig. 1A, when the RXRα expression vector was cotransfected with the reporter plasmid, we observed a strong induction of reporter gene transcriptional activity in response to 9-*cis*-RA but not to all-*trans*-RA, suggesting that activation of RXR by 9-*cis*-RA can positively regulate the transcriptional activity of the lactoferrin gene promoter. Cotransfection of the RARα expression vector slightly enhanced the CAT activity in response to both RAs. This induction was significantly enhanced when the RXRα expression vector was cotransfected. Consistent with previous observations (39), the reporter gene could be activated by estrogen (E_2) when the ER expression vector was present. These results therefore demonstrated that RAs and their receptors are involved in the regulation of lactoferrin gene promoter activity.

The transcriptional activation activity of RAs and their receptors is mainly mediated by response elements composed of AGGTCA or like sequences arranged as 1-, 2-, or 5-bp spacing (50, 65). We searched for the possible presence of RARE in the promoter region of the lactoferrin gene that could account for the transactivation activity of RAs and their receptors. Inspection of the sequence in the promoter region revealed the presence of two AGGTCA-like sequence motifs (−349 to −337) arranged as a direct repeat with 1-bp spacing similar to the one identified in the cellular retinol-binding protein type II (CRBP II) gene (46) (Fig. 1B). This type of sequence (DR-1) has been shown to confer RXR homodimer activity in response to 9-*cis*-RA (32a, 35, 70, 72). To examine whether this DR-1 element functions as an RARE, an oligonucleotide corresponding to the AGGTCA-like sequence motifs was synthesized and cloned into $pBLCAT_2$, which contains the thymidine kinase (tk) promoter and CAT gene (41). The resulting reporter was tested for its RA responsiveness by transient transfection in CV-1 cells. The $pBLCAT_2$ empty vector did not show any response to RAs and their receptors (data not shown). However, cotransfection of the lactoferrin-RARE-tk-CAT with the RXRα expression vector led to a strong induction of CAT activity in the presence of 9-*cis*-RA but not of all-*trans*-RA (Fig. 2A), suggesting that the DR-1 element can function as an RXR homodimer response element. Cotransfection of the RARα expression vector alone did not result in significant induction of reporter gene expression in response to either all-*trans*-RA or 9-*cis*-RA. However, when both the RARα and RXRα expression vectors were cotransfected, CAT activity was induced by either all-*trans*-RA or 9-*cis*-RA. The induction of RAR/RXR heterodimer activity by RAs may be due to an artificial RARE created by inserting multiple copies of lactoferrin-RARE into $pBLCAT_2$. Thus, our results are consistent with previous observations that DR-1 elements function as an RXR homodimer-specific RARE (32a, 46, 70). Interestingly, one of the lactoferrin-RARE core motifs is also used to form an ERE (Fig. 1B), which was previously characterized by Liu and Teng. (39). A synthetic oligonucleotide (−351 to −326) that contains composite RARE/ERE was then cloned into $pBLCAT_2$ and analyzed for its RA responsiveness in CV-1 cells. As shown in Fig. 2B, the lactoferrin composite RARE/ERE exhibited an RA-responsive pattern similar to that observed on the lactoferrin-RARE in that it was also strongly activated by RXR homodimers. As expected, the composite element was strongly activated by E_2 when the ER expression vector was cotransfected (Fig. 2B). Similar to previous observations on a consensus ERE (64), enhanced basal activity was observed on the lactoferrin-RARE/ERE when ER was present.

To ensure that the activation of the RARE/ERE by retinoid receptors was through the DR-1 element, point mutations were introduced into each half-site. CAT reporter genes containing one copy of each mutated oligonucleotide were tested in CV-1 cells for their responsiveness to RAs (Fig. 2C). Mutation of the 3′ half-site of the ERE (m1) did not show any clear effect on the transcriptional activity of retinoid receptors, whereas it completely abolished E_2-induced ER activity. Mutations in either half-site of the DR-1 element (m2 and m3) dramatically reduced 9-*cis*-RA-induced RXR homodimer activity, indicating that each half-site is functionally important for RXR homodimers. All-*trans*-RA- and 9-*cis*-RA-induced RAR/RXR heterodimer activity was also affected by these mutations, but to a lesser extent. This suggests that the DR-1 element may not mediate RAR/RXR heterodimer activity and also raises the possibility that other sequences, such as another putative half-site (as indicated by thin arrows in Fig. 1B), may contribute to the transactivation activity of RAR/RXR heterodimers. Further analysis may establish the involvement of these putative AGGTCA-like sequences.

To study the binding of retinoid and estrogen receptors to the composite RARE/ERE, gel retardation assays were carried out. As shown in Fig. 3, bacterially expressed RXR protein formed a strong complex with the DNA fragment. The binding

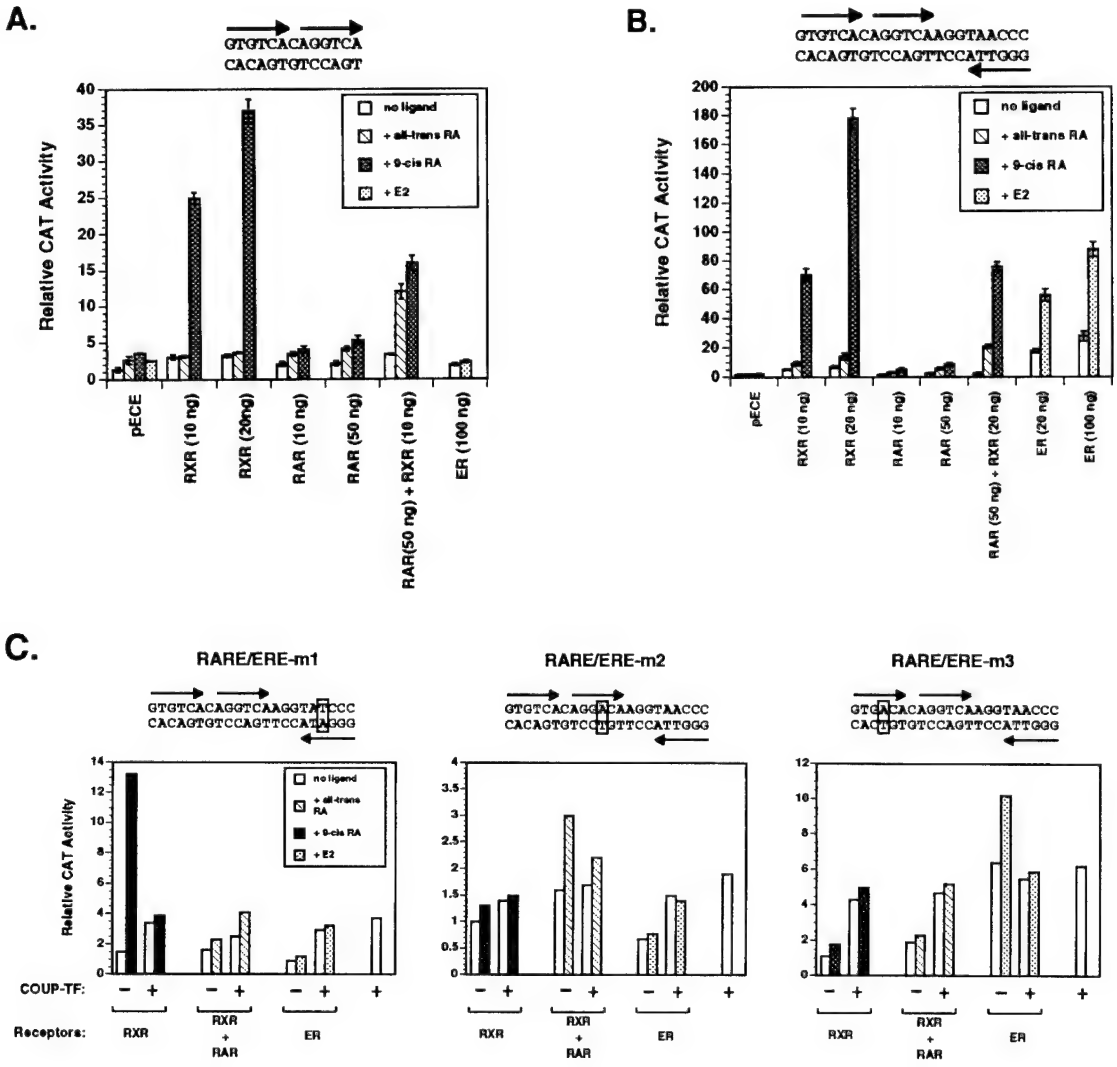

FIG. 2. Transactivational activity of the lactoferrin-RARE in CV-1 cells. (A and B) To determine the transactivation activity of the lactoferrin-RARE, CV-1 cells were transfected with (A) lactoferrin-RARE-tk-CAT or (B) lactoferrin-RARE/ERE-tk-CAT and the indicated amounts of RXRα, RARα, or ER expression vectors, treated with ligand (all-*trans*-RA or 9-*cis*-RA, 10^{-7} M; E_2, 10^{-8} M) for 24 h, and assayed for CAT activity. The results shown represent the means of three independent experiments. (C) Effects of mutations in the lactoferrin-RARE/ERE. Mutated lactoferrin-RARE/EREs (boxed nucleotides indicate mutated sequence) were cloned into $pBLCAT_2$. The resulting reporters were tested in CV-1 cells for their response to the indicated receptors and ligands.

of RXR was specific in that the complex could be specifically upshifted by anti-RXR antibody but not by nonspecific serum. Bacterially expressed RAR protein also showed binding with the composite element. When RXR and RAR were mixed, a prominent DNA-binding complex was observed, which could be upshifted by either anti-RXR or anti-RAR antibody. Consistent with previous observations (38), ER and COUP-TF orphan receptor formed strong complexes with the lactoferrin composite RARE/ERE. Together, these results demonstrate that the lactoferrin-RARE/ERE can interact with different nuclear hormone receptors.

COUP-TF orphan receptors repress retinoid and estrogen receptor activity on the lactoferrin-RARE/ERE composite response element. COUP-TF orphan receptor has been shown to inhibit ER activity as a result of its strong DNA binding to a region close to ERE (38). COUP-TF orphan receptor is also known to restrict retinoid response either through its high-affinity DNA binding to the RAREs or by its interaction with RXR (10, 28, 62, 67). In our gel retardation assays, COUP-TF orphan receptor bound to the lactoferrin-RARE/ERE with high affinity (Fig. 3). We therefore investigated whether COUP-TF orphan receptor could also repress retinoid receptor activity on the composite lactoferrin-RARE/ERE. In CV-1 cells, when COUP-TF orphan receptor was cotransfected, the 9-*cis*-RA-induced RXR homodimer activity on the reporter was markedly repressed (Fig. 4). The repression is very efficient in that the RA activity was almost completely inhibited when 2.5 ng of the COUP-TF expression vector was cotransfected. Consistent with previous observations (38), ER activity was also inhibited. Thus, the COUP-TF orphan receptor is an effective regulator of retinoid and estrogen activity on the lactoferrin gene promoter.

Functional interactions between retinoid and estrogen receptors on the lactoferrin-RARE/ERE. The finding that the

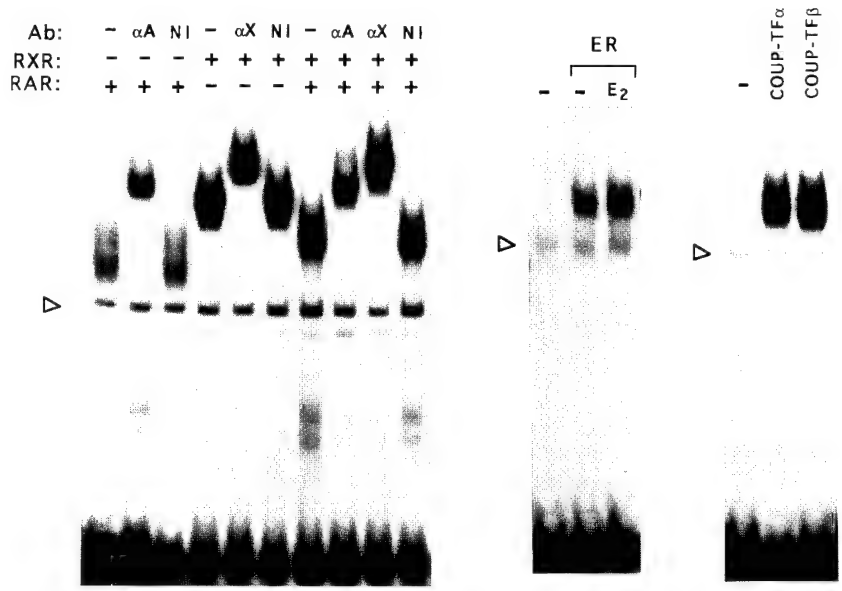

FIG. 3. Binding of lactoferrin-RARE/ERE with different nuclear hormone receptors. Bacterially expressed RXRα and RARγ (69, 70) (left panel) and in vitro-synthesized ER, COUP-TFα, and COUP-TFβ (right panels) were analyzed by gel retardation for their binding to the lactoferrin promoter region (−351 to −326). To determine the DNA-binding specificity, retinoid receptors were also incubated with antibodies (1 μl) against either RXR (αX) or RAR (αA) or nonspecific serum (NI) before electrophoresis. Open triangles represent nonspecific binding.

lactoferrin-RARE overlaps the lactoferrin-ERE and the observation that different nuclear hormone receptors can bind to the overlapped response element prompted us to investigate the interaction of different hormones and their receptors on the composite response element. As shown in Fig. 5A, 9-*cis*-RA-induced RXR homodimer activity was strongly inhibited by ER in a concentration-dependent manner. Cotransfection of 100 ng of ER expression vector led to a repression of about 60% of RXRα homodimer activity (Fig. 5A). Because of the high basal level of activity associated with ER (Fig. 2B) (64), further repression could not be observed when a larger amount of ER was used (data not shown). The inhibition is not due to a general squelching effect, since a higher reporter gene activity was obtained when both E_2 and 9-*cis*-RA were present. In contrast, E_2-induced ER activity was not clearly affected when the RXRα or RARα expression vector alone was cotransfected (Fig. 5B). However, cotransfection of both receptors resulted in about 50% repression of E_2-induced ER activity. Importantly, the high basal level of activity associated with ER was also markedly inhibited. Interestingly, RAR was not only required for RXR to repress ER activity but at same time also strongly inhibited 9-*cis*-RA-induced RXRα homodimer activity (Fig. 5C). The latter effect is likely due to titration of the RXR molecules from homodimer complexes to RXR/RAR heterodimer complexes, which are likely inactive on DR-1-like RARE (32a, 46). Thus, in this complex multiple-hormone-responsive system, RAR may play an important role in regulating both 9-*cis*-RA-induced RXR homodimer and E_2-induced ER activities.

Composite lactoferrin-RARE/ERE is activated by retinoid receptors in a cell type-specific manner. To further investigate the functional interaction between retinoids, their receptors, and ER on the composite element, transient-transfection assays were carried out in various breast cancer cell lines (Fig. 6). In ZR-75-1 cells, ER-positive cells, E_2 strongly induced reporter gene expression because of the presence of endogenous ER activity. About 10-fold induction was observed when the ER expression vector was cotransfected. In an ER-negative breast cancer cell line (Hs578T), induction of CAT activity by E_2 was only obtained when the ER expression vector was cotransfected. Surprisingly, when 9-*cis*-RA or all-*trans*-RA was used together with E_2, about 50 to 65% of E_2-induced ER activity was inhibited in both cell lines. Cotransfection of the RXR expression vector with ER also led to a slight inhibition (20 to 25%) of the E_2-induced ER activity in the absence of RA, but the inhibition was dramatic (85%) when 9-*cis*-RA was present. Similar results were obtained with other breast cancer cell lines (data not shown). Thus, repression of E_2 activity by

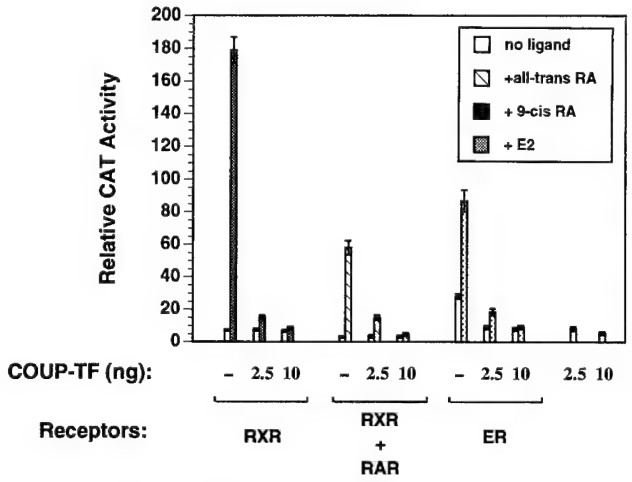

FIG. 4. COUP-TF orphan receptor represses retinoid receptor activity on the lactoferrin-RARE/ERE. The lactoferrin-RARE/ERE-tk-CAT reporter plasmid was cotransfected together with RARα (50 ng) and/or RXRα (20 ng) or ER expression vectors into CV-1 cells in the presence of the indicated amounts of COUP-TF expression vector (—, 0 ng). Transfected cells were not treated or treated with ligand (all-*trans*-RA or 9-*cis*-RA, 10^{-7} M; E_2, 10^{-8} M) and assayed 24 h later for CAT activity. Data shown represent the means of four independent experiments.

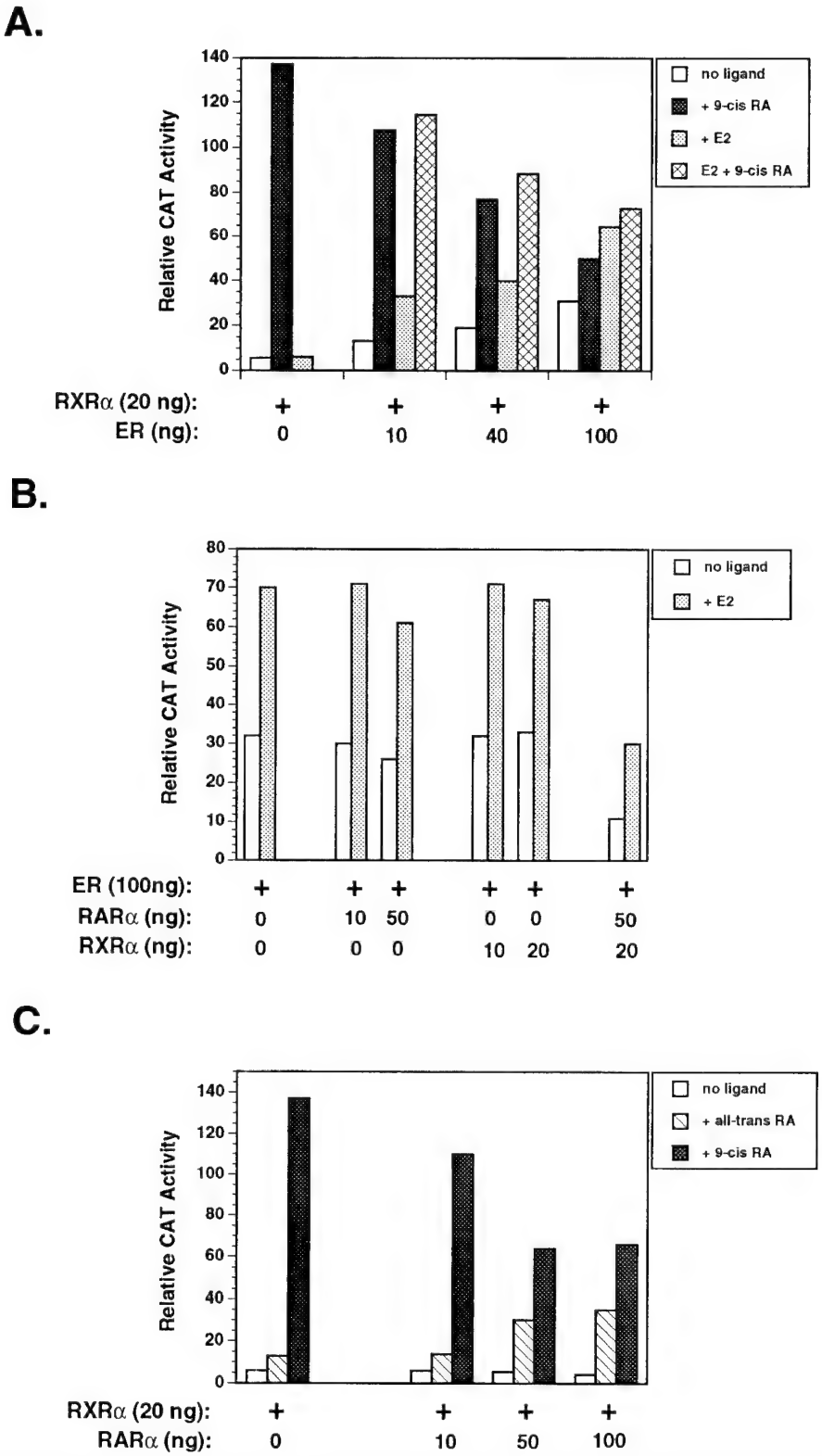

FIG. 5. Transcriptional activity of retinoid and estrogen receptors and their interaction on the lactoferrin-RARE/ERE in CV-1 cells. (A) Inhibition of RXR homodimer activity by ER. (B) Inhibition of ER activity by RAR/RXR heterodimers. (C) Inhibition of RXR homodimer activity by RAR. The lactoferrin-RARE/ERE-tk-CAT reporter plasmid was cotransfected with indicated amounts of ER, RARα, and/or RXRα into CV-1 cells. Transfected cells were not treated or treated with ligand (all-*trans*-RA or 9-*cis*-RA, 10^{-7} M; E_2, 10^{-8} M) or combinations of them and assayed 24 h later for CAT activity. Data shown represent the means of three independent experiments.

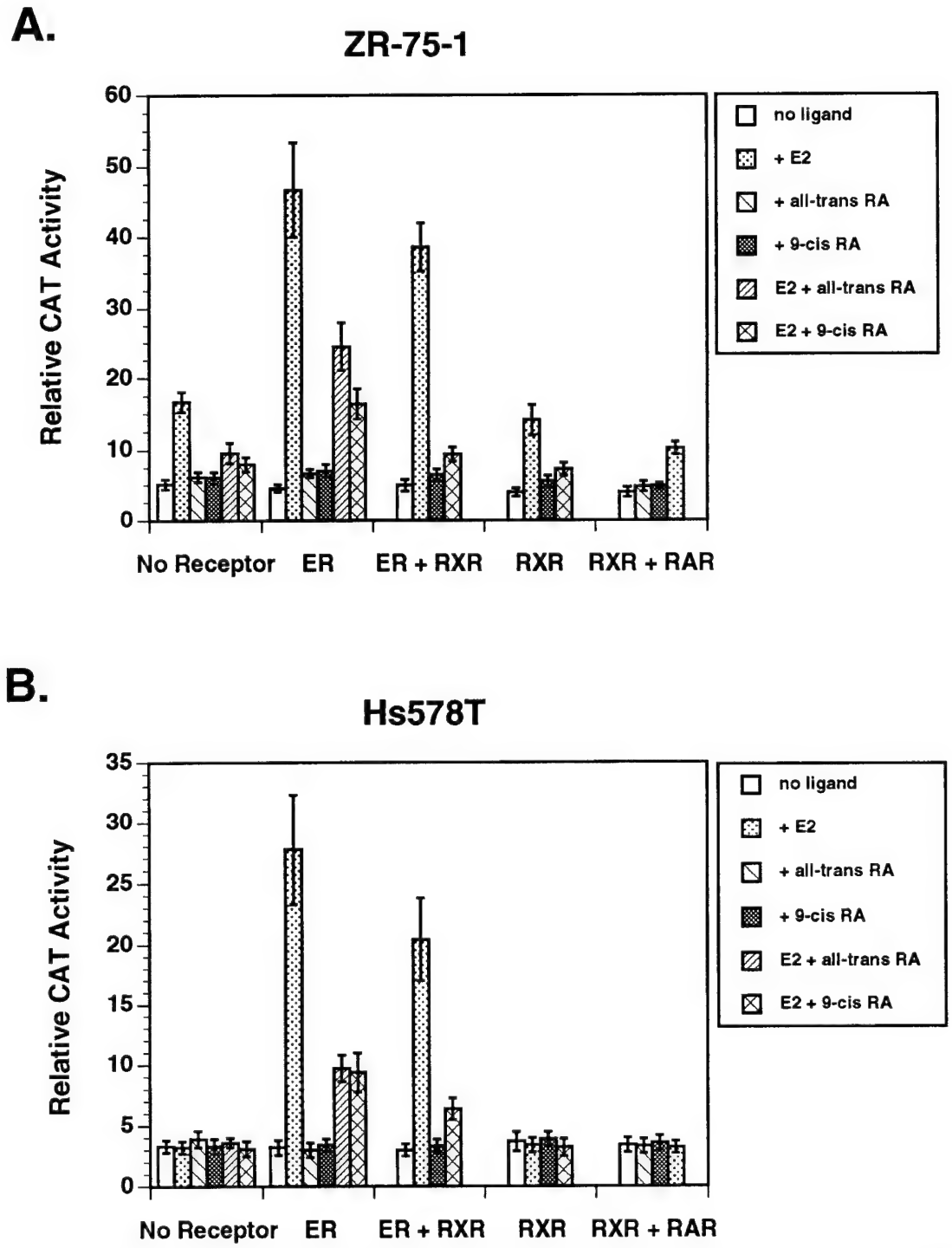

FIG. 6. Repression of estrogen-induced activation of the lactoferrin-RARE/ERE by RXR and RAs in breast cancer cells. The lactoferrin-RARE/ERE-tk-CAT reporter plasmid was cotransfected together with indicated combinations of ER (50 ng), RXRα (20 ng), or RARα (50 ng) expression vectors into ZR-75-1 (A) and Hs578T (B) cells. For comparison, the βRARE-tk-CAT reporter plasmid was cotransfected together with empty vector or RARα (50 ng) and/or RXRα (20 ng) expression vectors into ZR-75-1 (C) and Hs578T (D) cells. Transfected cells were not treated or treated with ligand (all-*trans*-RA or 9-*cis*-RA, 10^{-7} M; E_2, 10^{-8} M) or combinations of them, as indicated, and assayed 24 h later for CAT activity. Data shown represent the means of three independent experiments.

retinoid receptor in breast cancer cells is primarily RA dependent, and it is different from the observation made in CV-1 cells.

Although 9-*cis*-RA and all-*trans*-RA strongly inhibited ER activity in breast cancer cells, surprisingly, we did not observe any induction of CAT activity in response to both RAs even when the RXR and RAR expression vectors alone or together were cotransfected (Fig. 6). To test whether the loss of retinoid receptor activity on the lactoferrin-RARE/ERE is due to a general defect of RA response in breast cancer cells, the tran-

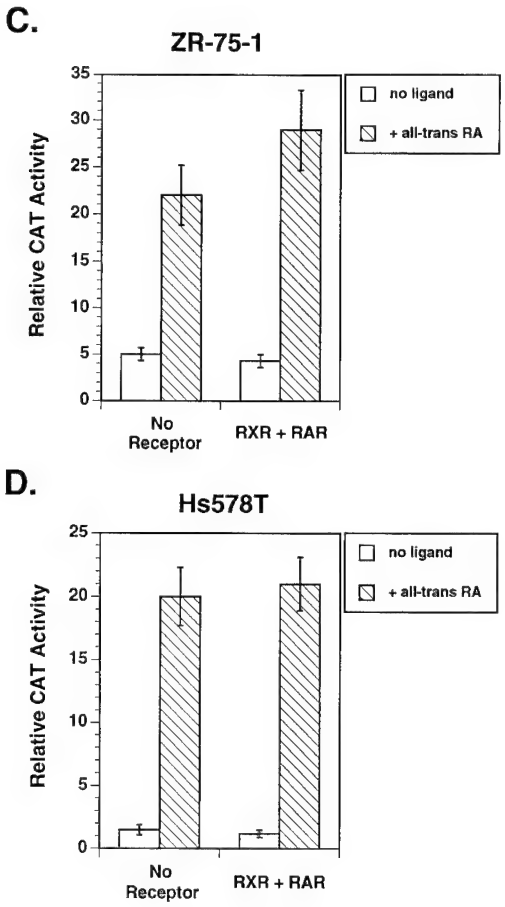

FIG. 6—*Continued.*

scriptional activity of another reporter that contains an RARE derived from the RARβ gene promoter (26) was examined. As shown in Fig. 6C and D, the endogenous retinoid receptors as well as cotransfected retinoid receptors in ZR-75-1 and Hs578T cells could strongly activate the reporter gene expression. Thus, the loss of RA response of the lactoferrin-RARE/ERE in breast cancer cells is not due to low levels of the endogenous retinoid receptors, and it appears to be lactoferrin-RARE/ERE specific.

To investigate whether the lactoferrin-RARE is functional in other cell types, the reporter construct containing the lactoferrin-RARE/ERE was introduced into HL-60 human promyelocytic leukemia cells, which undergo granulocytic differentiation in response to RA (7). As shown in Fig. 7A, the reporters were slightly activated by either all-*trans*-RA or 9-*cis*-RA. Further activation was observed when RAR or RAR and RXR expression vectors were cotransfected. Cotransfection of RXR/RAR heterodimers led to about twofold induction of reporter gene expression in response to either 9-*cis*-RA or all-*trans*-RA. A strong induction was observed when the RXR expression vector alone was cotransfected in the presence of 9-*cis*-RA but not of all-*trans*-RA. Similar results were obtained with another hematopoietic cell line, U-937 lymphoma cells (Fig. 7B). These data demonstrate that the lactoferrin-RARE/ERE can be activated by retinoid receptors in hematopoietic cells. Thus, the lactoferrin-RARE/ERE is differentially activated by retinoid receptors in different cell types.

COUP-TF is responsible for the differential transcriptional activities of the lactoferrin gene in different cell types. To investigate the possible molecular mechanism for the cell type-specific activation of the composite lactoferrin-RARE/ERE, nuclear proteins were prepared from HL-60 and U-937 cells and from breast cancer cells (ZR-75-1 and T-47D) and analyzed by gel retardation assay. Binding of the nuclear proteins to the SP-1 site was used as a control. As shown in Fig. 8A, nuclear proteins prepared from different cell lines displayed a similar binding pattern when the SP-1 binding site was used as a probe. However, nuclear proteins from HL-60 and U-937 cells exhibited very different DNA-binding complexes from those of the breast cancer cells when the lactoferrin-RARE/ERE was used. Two groups of high-molecular-weight complexes were observed with ZR-75-1 and T-47D cells. The binding of the high-molecular-weight complexes, in particular the slowest-migrating complex, was much stronger than that of nuclear proteins from HL-60 and U-937 cells. In addition, one group (the second slowest migrating) of the high-molecular-weight complexes formed with breast cancer cells migrated slightly more slowly than the corresponding complex observed with U-937 cells, suggesting that components of the binding complexes are different.

To determine the DNA-binding properties of these complexes, competition experiments were carried out (Fig. 8B and C). No clear effect was observed when the unlabeled lactoferrin-ERE (L-ERE) or consensus ERE or glucocorticoid response element (GRE) was used. In contrast, when the unlabeled DR-1-like element (L-DR-1) from the lactoferrin-RARE or the consensus DR-1 sequence (DR-1) was added, the binding of the high-molecular-weight complexes in U-937 nuclear proteins was completely inhibited. Although the binding complexes in ZR-75-1 were also dramatically inhibited by the DR-1, one of the complexes could not be inhibited even with an excess amount of the DR-1 oligonucleotide (Fig. 8D). This complex appears to bind to ERE, since addition of the ERE resulted in inhibition of its binding (last lane in Fig. 8C). Together, these data indicate that the DR-1 part is mainly responsible for the binding of the high-molecular-weight complexes.

To further determine the nature of these high-molecular-weight complexes, nuclear proteins were mixed with anti-RXR, anti-RAR, or anti-COUP-TF antibodies. The anti-RXR antibody used here recognizes all three RXRs (α, β, and γ) (unpublished observation). As shown in Fig. 8E, the binding of the second-slowest-migrating group of the high-molecular-weight complexes was dramatically affected by anti-RXR antibody but not by anti-RAR antibody. It was completely inhibited when nuclear proteins from ZR-75-1 cells were used, whereas it was only partially inhibited when nuclear proteins from U-937 cells were analyzed. These results indicate that binding complexes in this group contain RXR and further suggest that the components of the complexes formed by nuclear proteins from breast cancer cells are different from those formed by nuclear proteins from hematopoietic cells. Since one complex in this group formed by ZR-75-1 nuclear proteins represents ERE binding activity (Fig. 8C and D), the observation that it could be inhibited by anti-RXR antibody suggests that RXR is able to bind to ERE, probably through formation of a dimeric complex with an unknown nuclear protein. When anti-COUP-TF antibody (66) was used, the binding of the slowest-migrating complex from ZR-75-1 and U-937 cells was completely inhibited, demonstrating that the complex represents COUP-TF binding. Given the facts that the binding of this complex is much stronger in breast cancer cells than in hematopoietic cells (Fig. 8A) and that COUP-TF is an effective repressor of retinoid recep-

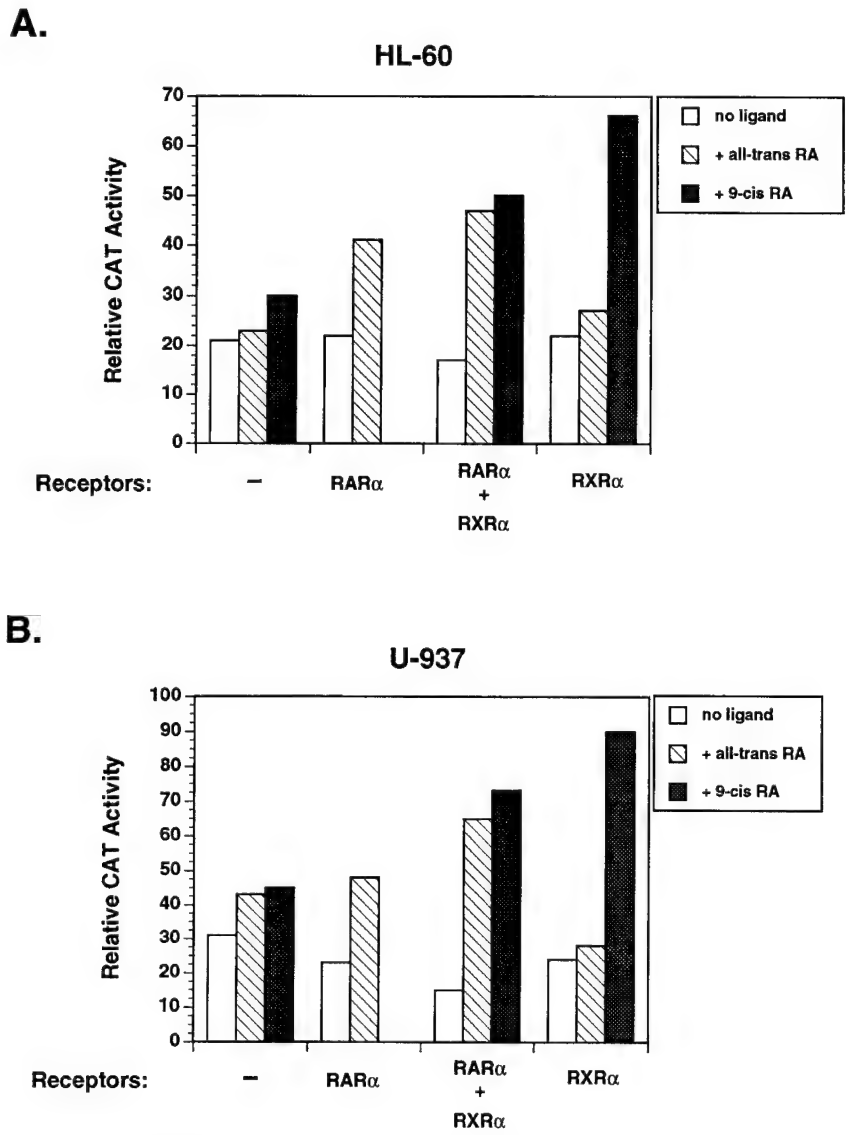

FIG. 7. Transactivation of the lactoferrin-RARE/ERE by retinoid receptors in HL-60 and U-937 cells. (A) Transactivation of lactoferrin-RARE/ERE by retinoid receptors in HL-60 cells. (B) Transactivation of lactoferrin-RARE/ERE by retinoid receptors in U-937 cells. The lactoferrin-RARE/ERE-tk-CAT reporter plasmid was cotransfected with empty vector or RARα (50 ng) and/or RXRα (20 ng) expression vectors into HL-60 and U-937 cells. Transfected cells were shocked with 10% glycerol, not treated or treated with the indicated ligand (all-*trans*-RA or 9-*cis*-RA, 10^{-7} M; E_2, 10^{-8} M), and assayed 24 h later for CAT activity. Data shown represent the means of two independent experiments.

tor activity (Fig. 5), this result allows us to conclude that COUP-TF is responsible for the differential transactivation activity of the lactoferrin-RARE/ERE observed in different cell types.

DISCUSSION

The lactoferrin gene is highly expressed in many different cell types, and its expression appears to be regulated very differently. An ERE located in the 5′-flanking region of the lactoferrin gene promoter has been identified and shown to mediate the transactivation activity of estrogen in mammary gland and female reproductive organs (39). However, the regulation of lactoferrin gene expression during the terminal differentiation of myeloid cells is largely unknown. In the present study, we have identified an RARE in the lactoferrin gene promoter. The lactoferrin-RARE is composed of two AGG TCA-like motifs arranged as a direct repeat with 1-bp spacing (Fig. 1B) and is strongly activated by RXR homodimers (Fig. 2). These results, together with the observation that the lactoferrin gene promoter is RA responsive in CV-1 cells (Fig. 1A), suggest that retinoids and their receptors are likely involved in the regulation of lactoferrin gene expression. Given the facts that RA is a potent inducer of the terminal differentiation process of myeloid cells (6, 7, 31, 63) and that the lactoferrin gene is only expressed at late stages of differentiation (18), our observation that the lactoferrin-RARE is functional in hematopoietic cells (Fig. 7) but not in human breast cancer cells (Fig. 6) points to the possibility that RA and its receptors may be involved in the regulation of lactoferrin gene expression during the terminal differentiation process of myeloid cells. At

present, the biological significance of such regulation is unclear.

Lactoferrin is known to have diverse functions. In neutrophils, lactoferrin was shown to inhibit myelopoiesis (8). Activation of lactoferrin gene expression by RA may represent a feedback regulatory mechanism which collaborates with other differentiation regulators to maintain the proper differentiation state of myeloid cells. Recently, it was reported that RA has both inhibitory and stimulatory effects on myelopoiesis (61). The positive regulation of lactoferrin gene expression by RA may therefore mediate the inhibitory effect of RA on myelopoiesis. Interestingly, lactoferrin has been found to be decreased or absent in most breast cancer and leukemia cells (51), suggesting that it may play a role in the malignancy process. This observation also implies that factors important in the regulation of lactoferrin gene expression may be abnormally expressed in these cells. In this context, we have found that the transcriptional activity of the lactoferrin gene promoter could not be activated by RAs and their receptors in human leukemia HL-60 and U-937 cells (data not shown), even though the promoter responded to RA in CV-1 cells (Fig. 1A). This result explains the lack of lactoferrin gene product in HL-60 cells (27) and further suggests that factors other than retinoid receptors are required to activate lactoferrin gene expression in hematopoietic cells.

One of the unique features of the lactoferrin-RARE is that it overlaps an ERE, in which both the RARE and ERE have a common AGGTCA-like motif (Fig. 1B). In addition, the region also contains two additional AGGTCA-like motifs that can form DR-0 elements (Fig. 1B) whose contribution is currently unknown. The composite response element binds to several different nuclear hormone receptor complexes, including RXR homodimers, RAR/RXR heterodimers, ER, and COUP-TF orphan receptors (Fig. 3). Our in vitro transactivation studies demonstrate that retinoid and estrogen receptors are involved in the positive regulation of the lactoferrin-RARE/ERE, while the COUP-TF orphan receptor functions mainly as a negative regulator of the element to restrict retinoid and estrogen responses. Thus, the lactoferrin composite element may serve as a tool for the lactoferrin gene promoter to respond properly to various physiological messages in different cell types (see below). Such a composite element may provide a basis for the involvement of retinoids and their receptors in the versatile hormonal signaling network and may explain the diverse biological functions of retinoids. Similar types of composite response elements have been observed in the regulatory regions of many other genes and are known to provide combined interactions between different regulatory networks (see reference 11 for a review). For example, a composite element in the promoter of the rat oxytocin gene can mediate retinoid, thyroid hormone, and estrogen signals and their collaboration and confer cell-specific expression of the gene (1), while a 25-bp composite response element found in the promoter region of the proliferin gene allows interaction between AP-1 and steroid hormone signals (13, 52). Similarly, the vitamin D response element (VDRE) in the human osteocalcin gene overlaps an RARE and an AP-1 binding site (59). Thus, distinct regulatory systems are able to converge into a common element for the fine control of gene expression.

Retinoids are known to interact with sex hormone signals. They can inhibit E_2-induced proliferation of breast cancer cells and are considered to be antiestrogen (15, 30). However, how retinoids act to antagonize estrogen activity is unclear. The present finding that the lactoferrin-RARE and lactoferrin-ERE overlap reveals a mechanism by which retinoid and estrogen signals interact. In CV-1 cells, when both ER and retinoid receptors were present, they interfered with each other's activity (Fig. 5). The observation that retinoid receptors and ER can bind to the overlapped DNA sequences suggests that competition for a DNA-binding site is likely the main mechanism by which retinoid and estrogen signals interact in CV-1 cells. Such a mechanism is widely utilized by other transcriptional factors (11, 12, 19, 20, 73) and is probably also used by COUP-TF orphan receptor to repress retinoid and estrogen activities on the lactoferrin-RARE/ERE (Fig. 4). Interestingly, RAR could also inhibit RXR homodimer activity (Fig. 2 and 5). Since RAR can interact with RXR in solution (36, 69) and RXR/RAR heterodimers are not effective transcriptional activators on the lactoferrin-RARE (Fig. 2), the titration of RXR from RXR homodimers by RAR is most likely responsible for this inhibition. A similar competition between RXR heterodimer and homodimer, which results in inhibition of each other's activity, has been observed previously (35, 46). Thus, it is expected that RAR plays a critical role in regulating lactoferrin gene expression in response to both retinoids and estrogen.

In contrast to the observation made with CV-1 cells, an RA-dependent inhibition of ER activity was found in breast cancer cells (Fig. 6). In this case, the transactivation activity of ER was strongly inhibited by retinoids even in the absence of cotransfected retinoid receptors. Thus, a different mechanism may be employed in breast cancer cells that allows RA and estrogen signals to interact. How RA represses ER activity is currently unknown. The fact that cotransfection of retinoid receptors enhanced the inhibitory effect (Fig. 6) suggests that the inhibitory effect of RA is mediated by endogenous retinoid receptors. RA may promote DNA binding of the retinoid receptor-containing complexes to the ERE or DR-1 site and therefore interfere with ER activity. The observation from our gel retardation assays (Fig. 8) that RXR, most likely complexed with another nuclear protein, can bind to the ERE suggests that the binding of the RXR-containing complex to the ERE may contribute to the inhibitory effect of RA. This is supported by our recent observation that a DNA sequence restricted to an ERE is sufficient to mediate the RA effect (37a). A similar result was reported in a recent study (60) showing that RXRβ could antagonize ER activity by the formation of an ERE binding complex with a protein present in breast cancer cells. Identification of the new RXR heterodimerization partner will enhance our understanding of the mechanism by which retinoid and estrogen signals interact in breast cancer cells.

Our transient-transfection experiments demonstrate that the transactivation of lactoferrin-RARE by retinoid receptors is cell type specific. The lactoferrin-RARE was highly activated by RAs in CV-1 cells (Fig. 2) and could also be activated in hematopoietic cells (Fig. 7). In contrast, neither RXR homodimers nor RAR/RXR heterodimer activity was observed in breast cancer cells even in the presence of cotransfected receptors (Fig. 6). The loss of retinoid receptor activity could not be explained by a decreased expression level of retinoid receptors or failure of retinoid uptake, since strong repression of ER activity and induction of the βRARE activity by RA were observed under the same conditions (Fig. 6). Thus, the loss of retinoid receptor activity on the lactoferrin-RARE/ERE in breast cancer cells is specific to this response element. The fact that the lactoferrin-RARE/ERE could be activated by estrogen in breast cancer cells (Fig. 6) suggests that the composite element may function mainly to confer ER activity in breast or other female reproductive tissues, and RA may act in these tissues to regulate ER activity. In contrast, the composite element may mainly mediate the retinoid effect in hematopoi-

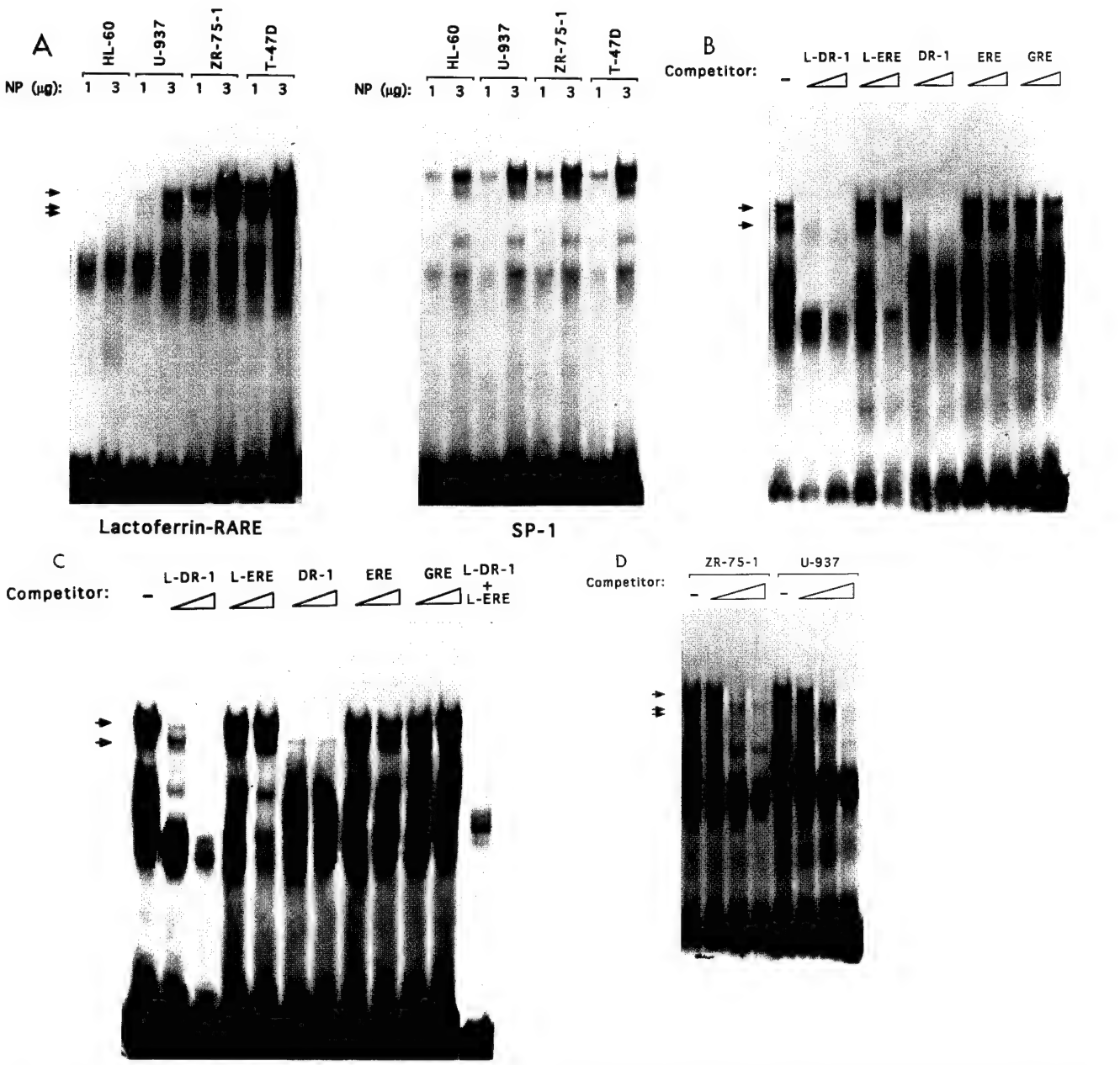

FIG. 8. Binding of nuclear proteins prepared from breast cancer or hematopoietic cells to the lactoferrin composite hormone response element. (A) Binding of nuclear proteins (NP) prepared from hematopoietic cells (HL-60 and U-937) or breast cancer cells (ZR-57-1 and T-47D) to the lactoferrin-RARE/ERE or SP-1 binding site. The indicated amounts of nuclear proteins prepared from different cell lines were analyzed for their binding to the lactoferrin-RARE/ERE composite element or SP-1 binding site by a gel retardation assay with labeled lactoferrin-RARE/ERE or SP-1 binding site as a probe. Arrows indicate the high-molecular-weight binding complexes. (B and C) Competition with binding of the high-molecular-weight complexes by the hormone response element. To determine the binding nature of the high-molecular-weight complexes, nuclear proteins prepared from U-937 (B) and ZR-75-1 (C) cells were incubated with two different concentrations (10 and 100 ng) of unlabeled hormone response element: lactoferrin-RARE (L-DR-1), lactoferrin-ERE (L-ERE), consensus DR-1 (DR-1), consensus ERE (ERE), or GRE for 5 min at room temperature before performance of the DNA-binding assay. Arrows indicate the high-molecular-weight binding complexes. (D) Comparison of the high-molecular-weight binding complexes from ZR-75-1 and U-937 cells. To determine the affinity and nature of the high-molecular-weight binding complexes observed with ZR-75-1 and U-937 cells, nuclear proteins prepared from ZR-57-1 (1 μg) and U-937 (3 μg) cells were incubated with different concentrations (1, 10, and 100 ng) of unlabeled lactoferrin-RARE for 5 min at room temperature before performance of the DNA-binding assay with labeled lactoferrin-RARE/ERE as a probe. Arrows indicate the high-molecular-weight binding complexes. (E) Effects of anti-RXR (αRXR), anti-RAR (αRAR), and anti-COUP-TF antibodies on the binding of the high-molecular-weight binding complexes. Nuclear proteins from ZR-75-1 (1 μg) and U-937 (3 μg) cells were incubated with antibody (Ab) (1 μl) for 30 min at room temperature before performance of the DNA-binding assay. Arrows indicate the high-molecular-weight binding complexes. The open triangle indicates the COUP-TF binding complex, and the solid triangle shows the upshifted COUP-TF complex.

etic cells. Thus, the lactoferrin composite hormone response element may serve as a major signaling switch module that allows different hormonal sensitivities in different cell types.

In the course of the investigation on the molecular mechanism by which the hormonal signaling switch is operated in the lactoferrin-RARE/ERE, we found that nuclear proteins from breast cancer cells formed much stronger complexes with the composite element than those prepared from HL-60 or U-937

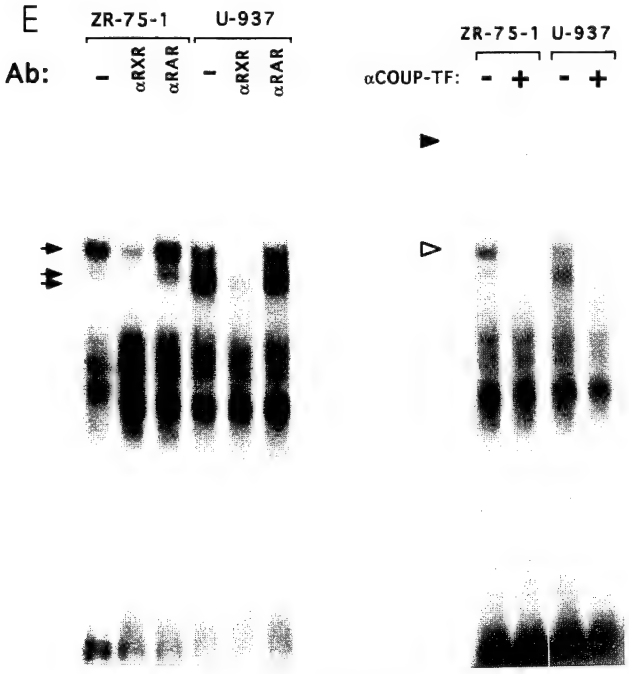

FIG. 8—*Continued.*

cells (Fig. 8A). Some of these strong DNA-binding complexes contain RXR, as judged from the results of the anti-RXR antibody study. These RXR-containing complexes on the lactoferrin-RARE/ERE observed with nuclear proteins from breast cancer cells appear to be transactivationally inactive, since RAs could not activate the element (Fig. 6). Therefore, the strong DNA-binding complexes on the lactoferrin-RARE/ERE in breast cancer cells may interfere with RXR homodimer and heterodimer DNA binding and thereby contribute to the loss of retinoid activity in these cells. Using anti-COUP-TF antibody, we found that the major DNA-binding complexes present in breast cancer cells contained COUP-TF (Fig. 8E). The COUP-TF bound specifically to the DR-1 part of the composite element, as revealed by our competition studies (Fig. 8B to D), consistent with previous observations (38). Nuclear proteins from breast cancer cells exhibited a much stronger COUP-TF binding complex (the slowest-migrating complex) than those prepared from hematopoietic cells (Fig. 8A). Such strong binding of COUP-TF may allow effective repression of retinoid response in breast cancer cells. A similar observation has recently been made in human endometrium carcinoma RL 95-2 cells, in which COUP-TF was suggested to be responsible for the downregulation of lactoferrin gene expression (39). COUP-TF may play a major role in the negative regulation of lactoferrin gene expression through its strong DNA binding to the composite element. However, such binding may not be sufficient to completely restrict estrogen activity, since the composite element could be activated by E_2 in breast cancer cells (Fig. 6). Interestingly, COUP-TF could not inhibit RA response on the βRARE (Fig. 6C and D). This is consistent with our previous observation in CV-1 cells (62). In this case, cotransfection of COUP-TF strongly inhibited retinoid receptor activity on the CRBP-II-RARE and ApoAI-RARE, which are composed of AGGTCA motifs arranged as a direct repeat with 1- or 2-bp spacing, respectively, whereas it had no effect on the βRARE (62). These observations and the present results obtained with breast cancer cells strongly suggest that COUP-TF may function to restrict RA response to DR-1- and DR-2-type RAREs. Together, our studies demonstrate that COUP-TF plays a major role in the signaling switch of the lactoferrin composite element and is likely responsible for the differential expression of the lactoferrin gene in different cell types.

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ABSTRACT

Retinoids are known to inhibit the growth of hormone-dependent but not of hormone-independent breast cancer cells. We investigated the involvement of retinoic acid receptors (RARs) in the differential growth inhibitory effect of retinoids and the underlying mechanism. Our data demonstrate that induction of RARβ by RA correlates with the growth inhibitory effect of retinoids. The hormone-independent cells acquired RA sensitivity when RARβ expression vector was introduced and expressed in the cells. In addition, RA sensitivity of hormone-dependent cells was inhibited by a RARβ-selective antagonist and the expression of RARβ anti-sense RNA. Introduction of RARα was also capable of restoring RA sensitivity in hormone-independent cells, but through induction of endogenous RARβ expression. Furthermore, we show that induction of apoptosis contributes to the growth inhibitory effect of RARβ. Thus, RARβ can mediate retinoid action in breast cancer cells by promoting apoptosis. Loss of RARβ, therefore, may contribute to the tumorigenicity of human mammary epithelial cells.

INTRODUCTION

Retinoids, the natural and synthetic vitamin A derivatives, are known to regulate a broad range of biological processes, including growth, differentiation and development (27, 49, 65). They are currently used in the treatment of epithelial cancer and promyelocytic leukemia and are being evaluated as preventive and therapeutic agents for a variety of other human cancers (27, 48, 49, 65). The effects of retinoids are mainly mediated by two classes of nuclear receptors; the retinoic acid receptors (4, 6, 24, 41, 64) and the retinoid X

receptors (RXRs) (28, 44, 50, 51, 82). RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily that also includes receptors for estrogen and vitamin D (18, 26). Both types of retinoid receptors are encoded by three distinct genes, α, β, and γ. These receptors display distinct patterns of expression during development and differentiation (27), suggesting that each of them may have specific function. All-trans RA and 9-cis RA (29, 46), the two known active derivatives of vitamin A, essentially function as hormones by interacting with specific retinoid receptors. All-trans RA binds and activates RARs, and 9-cis RA is capable to bind and activate both RARs and RXRs. For transactivation, RARs require interaction with RXRs (7, 38, 44, 54, 82, 86), resulting in the formation of RAR-RXR heterodimers that recognize RA response elements (RAREs) located in the regulatory regions of target genes. RXR can also heterodimerize with several other nuclear hormone receptors (10, 52, 83 for review). In addition, in the presence of 9-cis RA, RXR can function as homodimers (84, 85) that bind to a set of distinct RAREs, and mediate a different response pathway. Some of the target genes are RARs themselves (15, 30, 43, 45, 72), in particular the RARβ gene where a RARE (βRARE) was identified in its promoter region that mediates the RA-induced RARβ gene expression (15, 30, 72). Auto-regulation of RARβ gene presumably plays a critical role in amplifying RA response.

Altered nuclear receptor activities can be associated with carcinogenesis. A well-known example is the v-erbA, a mutated form of thyroid hormone receptor (TR) (12). In human acute promyelocytic leukemia (APL) cells, an abnormal RARα transcript is produced by chromosomal translocation (13, 37). The involvement of RARβ in cancer development was originally implicated in the finding that it was integrated by hepatitis B virus in human hepatoma (14). Recently, it was found that RARβ was not expressed in a number of malignant tumors including lung carcinoma, squamous cell carcinoma of the head and neck, and breast carcinoma (23, 31, 33, 62, 73, 87). Given the fact that retinoids are key players in the regulatory network of cell differentiation and proliferation (27, 49, 65),

altered retinoid receptors can result in abnormal cellular differentiation pathways and a loss of their anti-proliferating effect, such as anti-AP-1 activity (68, 80). Recently, several studies have reported that retinoids can induce apoptosis in several different cell types (60, 63, 69). Apoptosis, a programmed cell death, is an important physiologic process in normal development and tissue homeostasis (20, 75). Since apoptosis is an autonomous suicide pathway that restricts cell numbers, induction of apoptosis by retinoids may represent an important mechanism by which retinoids inhibit cancer cell growth. Alteration of retinoid receptor activity may therefore lead to suppression of apoptosis and result in pathological accumulation of aberrant cells and disease such as tumor.

Breast cancer is the most common cancer among women. Epidemiological, experimental and clinical studies indicate that hormones play a major role in the etiology of the cancer. Accordingly, endocrine treatment, in particular the administration of anti-estrogen, is commonly used. Unfortunately, hormone dependence is often lost upon progression of the disease, resulting in a more aggressive hormone-independent tumor (11, 48). A considerable volume of human and animal data have suggested retinoids as novel agents for the prevention and treatment of breast cancer (11, 34, 58). In animals, administration of retinoids inhibits the initiation and promotion of mammary tumors induced by carcinogens (11, 58). In vitro, retinoids have been shown to inhibit the growth of human breast cancer cells (21, 22, 40, 55, 67, 70, 71, 74, 76-78). Growth inhibition of such cells in culture has been observed when retinoids are administered alone or in combination with other agents, such as anti-estrogen (21, 40) or interferon (79), where synergistic effects have been observed. Based on these results, several clinical trials with retinoids have been carried out (5, 9, 57). Unfortunately, these early clinical trials on patients with advanced breast cancer have not demonstrated significant effort, except some benefits were observed when retinoids were used together with anti-estrogen (5).

The observations that retinoids are effective in the prevention of breast cancer development and that the activity is lost in patients with advanced breast cancer suggest a loss of retinoid sensitivity during progression of breast tumor. This is also demonstrated by in vitro observations that the growth inhibitory effects of retinoids are mainly seen in hormone-dependent ER positive breast cancer cells and that hormone-independent ER negative cells are refractory to the retinoid effect (21, 76). How retinoids inhibit the growth of hormone-dependent breast cancer cells and how their inhibitory effect is lost in hormone-independent cells remain largely unclear. Since the growth inhibitory effects of retinoids are likely mediated by their nuclear receptors, several studies (66, 76) have examined the expression of retinoid receptor genes in a number of hormone-dependent and -independent breast cancer cell lines. These studies have consistently revealed an equal level expression of RARγ in all hormone-dependent and -independent cell lines as well as several normal human mammary epithelial lines investigated, suggesting that RARγ is unlikely involved in the differential growth inhibitory effect of RA on breast cancer cells. RARα was expressed in most of the cancer cell lines (66) but relative low expression levels of its transcripts were observed in some of the hormone-independent cell lines. In the case of RARβ, its expression levels were variable among the cell lines (66, 76). These data, therefore, could not provide a clear explanation for the differential sensitivities of breast cancer cell lines to retinoids. Since the expression of RAR genes (15, 30, 43, 45, 72), especially the RARβ gene (15, 30, 72) is known to be regulated by RA and since the expression of RARs previously reported (66, 76) was not well examined in response to RA, the results obtained may not reflect the real levels of RARs, that determine RA responsiveness. To further study the involvement of retinoid receptors, we have determined the expression of RARs in a number of hormone-dependent and -independent breast cancer cell lines in the absence and in the presence of RA. Our results demonstrated that the expression of RARβ gene was dramatically induced by RA in hormone-dependent but not in hormone-independent breast cancer cell lines. Induction of RARβ by RA correlated with the growth inhibitory

effect of retinoids in the cell lines investigated. The requirement of RARβ expression for the RA-induced growth-inhibition was further demonstrated by restored RA sensitivities in hormone-independent cells after introduction of RARβ and diminished RA responses in hormone-dependent cells by a RARβ selective antagonist and the expression of RARβ anti-sense RNA. In addition, our data demonstrated that RARβ could promote apoptosis in breast cancer cells. Thus, the loss of RARβ gene expression could be one of the major factors responsible for the loss of RA sensitivity in breast cancer cells and may contribute to their transformed phenotype.

MATERIALS AND METHODS

Cell culture. Breast cancer cell lines, ZR-75-1, T-47D, MB231, BT-20, and MB468 were obtained from American Type Culture Collection (ATCC). MCF-7 was obtained from Dr. S. Sukumar (Salk Institute, La Jolla, CA). ZR-75-1 and T-47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS); MB231, MCF-7 and MB468 cells were grown in DME medium supplemented with 10% FCS; BT-20 cells were maintained in MEM medium with 10% FCS.

Growth inhibition assay. To study anchorage-dependent growth, cells were seeded at 1,000-2,000 cells per well in 96-well plates, and treated with various concentrations of retinoids. Media were changed every 48 hr. The number of viable cells were determined by their capacity to convert a tetrazolium salt into a blue formazan product using a non-radioactive Cell Proliferation/Cytotoxicity Assay Kit (Promega, Madison, WI) (59). For anchorage-independent growth assay, 30,000 cells/60-mm dish in culture medium containing 10% FCS, 0.3% agar (Difco, Detroit, MI) and 10^{-7} M RA were plated onto an already hardened 0.6% agar underlayer in medium supplemented with 10% FCS. The

plates were incubated for 21 days in 5% CO_2 incubator. Colonies with more than 40 cells were scored as positive.

RNA preparation and Northern blot. For Northern blot analysis, total RNAs were prepared by guanidine hydrochloride/ultracentrifugation method (53). About 30 μg total RNAs from different cell lines were fractionated on 1% agarose gel, transferred to Nylon filters and probed with the ^{32}P-labeled ligand binding domain of RAR cDNAs as previously described (87). To determine that equal amounts of RNA were used, the filters were also probed with ribosomal RNA L32 cDNA.

Preparation of nuclear extracts and gel retardation assays. Nuclear extracts were prepared essentially according to the method previously described (42). Briefly, cells growing in about 90% confluence were washed with cold phosphate buffered saline (PBS) and scraped into PBS using a rubber policeman. Cells were pelleted by low speed centrifugation and then resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 3 mM $CaCl_2$ and 2 mM $MgCl_2$. After pelleting, the cells were lysed in the buffer containing 1% NP-40 by 10 to 15 strokes using ice-cold Dounce homogenizer. Immediately after lysis, nuclei were collected by centrifugation at 2000 x g and washed once with a buffer containing 10 mM Hepes-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol. Nuclear protein were extracted with a high salt buffer containing 20 mM Hepes-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol. All the buffers used for the procedure contained protease inhibitors, *i.e.*, 100 μg/ml PMSF, 1 μg/ml leupeptin and 1μg/ml aprotinin. When it was necessary, nuclear extracts were concentrated by centricon 10 (Millipore). Small aliquots of nuclear proteins were immediately frozen and kept at -80°C until use. To study βRARE binding, nuclear extracts were prepared from different breast cancer cell lines treated with or without 10^{-6} M RA. 5 μg of nuclear extracts from different breast cancer cells were analyzed by gel

retardation assay for their βRARE binding activity using ^{32}P-labeled βRARE as a probe as described previously (42). The βRARE used in the experiments is the direct repeat of RA response element present in RARβ promoter (AGGGTTCAGGCAAAGTTCAC). Labeled DNA probes were purified by gel electrophoresis and used for the gel retardation assay.

Transient transfection and CAT assay. To measure transcriptional activation of βRARE in breast cancer cell lines, βRARE linked with CAT gene (βRARE-tk-CAT) was used as reporter genes to determine RA response in indicated hormone dependent and independent human breast cancer cell lines. 2.0 μg of βRARE-tk-CAT, and 3.0 μg of β-gal expression vector (pCH 110, Pharmacia) were transiently transfected into the indicated cells using the calcium phosphate precipitation method (42). Cells were grown in the presence or absence of 10^{-7} M RA. Transfection efficiency was normalized by β-gal activity. The data shown are means of 3 separate experiments.

Stable transfection. To construct RARβ and RARα expression vector, cDNA for the RARβ or RARα gene was cloned into pRc/CMV expression vector (Invitrogene, San Diego, CA). To construct RARβ anti-sense expression vector, cDNA for the RARβ gene was cloned into pRc/CMV expression vector in an anti-sense orientation. The resulting recombinant constructs were then stably transfected into breast cancer cells using calcium phosphate precipitation method, and screened using 400 μg G418 (Gibco BRL, Grand Island, NY). The integration and expression of exogenous RARβ and RARα cDNA were determined by Southern blot and Northern blot, respectively.

Apoptosis analysis. For morphological analysis (35), 10^{-6} M RA treated or untreated cells were trypsinized, washed with PBS. After fixation with 3.7% paraformaldehyde followed by acid-alcohol treatment, cells were stained with 50 μg/ml propidium iodine (PI) containing 100 μg/ml DNase-free RNase A to visualize the nuclei. Stained cells were examined with a Zeiss LSM 410 confocal laser-scanning microscopy. Overlays of cells

were made with confocal sections at increment of 1μm. Apoptotic nuclei were condensed and were more brightly stained than non-apoptotic ones. For TUNEL assays (25), cells were treated with or without 10^{-6} M RA. After 24 hours, cells were trypsinized, washed with PBS, and fixed in 1% formaldehyde in PBS (pH 7.4). After washing with PBS, cells were resuspended in 70% ice-cold ethanol and immediately stored at -20°C for overnight. Cells were then labeled with biotin-16-dUTP by terminal transferase and stained with avidin-FITC (Boehringer Mannheim, Germany). Fluorescence labeled cells were analyzed using a FACScater-Plus. Representative histograms were shown. For ELISA assay, breast cancer cell MB231, MB231/RARβ3, ZR-75-1, and ZR-75-1/A-RARβ10 were split at the same time and treated with 10^{-6} M RA for 12, 24 and 48 hours. Treated cells were harvested at the same time. DNA fragmentation was measured with the use of the Cell Death Detection ELISA kit (Boehringer Mannheim, Germany). About 2 X 10^4 cells were assayed for DNA fragmentation following manufacture's protocol. The results are expressed relative to RA untreated controls.

RESULTS

Activation of RARs but not RXRs is responsible for RA-induced growth inhibition in breast cancer cells.

Retinoids are known to inhibit the growth of breast cancer cells. However, how the growth inhibitory effect of retinoids is mediated is largely unknown. The effects of retinoids are mainly mediated by two classes of retinoid receptors, the RARs and the RXRs, that function as either RAR/RXR heterodimers or RXR homodimers (10, 52, 83). All-trans RA can activate both dimeric complexes due to its isomerization to 9-cis RA in cells (29, 46), while 9-cis RA can bind and activate both RARs and RXRs. To establish the involvement of RAR and RXR in RA-induced growth inhibition in hormone-dependent

breast cancer cells, we used retinoids selective for RXR homodimers and RXR/RAR heterodimers. Ch55 (36) and (all-E)-**UAB8** (1), which specifically bind RARs and activate RXR/RAR heterodimers, displayed a similar degree of growth inhibition, as that observed with all-trans RA in ZR-75-1 and T-47D cells, while (9Z)-**UAB8** (1) which specifically activates RXR homodimers did not show clear growth inhibitory effects (figure 1). These data suggest that activation of RARs is mainly responsible for the RA-induced growth inhibition of breast cancer cells, consistent with a previous observation (70).

Induction of RARβ by RA correlates with the growth inhibitory effect of RA.

To determine which RAR subtype is involved in RA-induced growth inhibition, we investigated the expression of three types of RARs (α, β, and γ) in a number of human breast cancer cell lines, including hormone-dependent (T-47D, ZR-75-1, and MCF-7) and hormone-independent (MB-468, BT-20, and MB231) lines. Similar to previous observations (66, 76), transcript for RARγ was detected in all of the cell lines with a similar expression level (figure 2a). RARα transcripts were also present in all of the cell lines. However, relative low levels of RAR expression were found in two hormone-independent cell lines (MB231 and MB-468) (figure 2b). All of these cell lines did not exhibit detectable levels of RARβ mRNA under the conditions used (figure 2c). These expression data did not show a clear correlation with RA-induced growth inhibition observed in these cell lines (figure 2d). Since the expression of RARs could be regulated by RA due to the presence of RAREs in their promoter regions (15, 30, 43, 45, 72), we analyzed the expression of RARs in the presence of RA in these cell lines. Treatment of these cells by 10^{-6} M all-trans RA for 36 hours did not show any effect on the expression levels of RARα and RARγ (figure 2a, b). However, the expression of RARβ was strongly enhanced by RA in the hormone-dependent cell lines (figure 2c). Surprisingly, RA failed to induce RARβ in hormone-independent cell lines. When the growth inhibitory effect of all-trans

RA and 9-cis RA was examined, both RAs showed a strong growth inhibition on hormone-dependent cell lines while they had little effect on hormone-independent lines (figure 2d). Thus, the induction of RARβ gene expression by RA correlates with RA-induced growth inhibition, suggesting that RARβ may mediate the differential growth inhibitory effects of RA in breast cancer cells.

Abnormal transcriptional regulation of βRARE in hormone-independent breast cancer cells.

RA-induced RARβ expression is mediated by the βRARE present in the RARβ promoter (15, 30, 72). The loss of RA response in inducing RARβ gene expression in hormone-independent human breast cancer cell lines indicates that the regulation of RARβ expression by RA is disturbed in these cells. To further examine the impaired RA response, a CAT reporter construct containing the βRARE linked with thymidine kinase (tk) promoter (βRARE-tk-CAT) (30) was used as a reporter to determine the degrees of RA response in both hormone-dependent and -independent cancer cell lines by transient transfection assays. When this reporter was transfected into hormone-dependent cells (T47D and ZR-75-1), a strong induction of CAT activity in response to RA was observed (figure 3a). In contrast, only slight induction of the CAT gene expression was seen in hormone-independent cells (MB231 and MB468). These results are consistent with a previous observation (76) and suggest that the loss of RARβ expression in hormone-independent breast cancer cells may be due to an abnormal transcriptional regulation of the βRARE. To investigate whether the loss of the βRARE activity is due to altered βRARE binding, nuclear proteins were prepared from hormone-dependent and -independent breast cancer cells treated either with or without RA and analyzed by gel retardation for their binding to the βRARE. As shown in figure 3b, a strong DNA binding complex was observed when nuclear proteins prepared from ZR-75-1 or T47D cells were used. The binding of this complex was much stronger when nuclear proteins were prepared from cells treated with

RA. However, this complex was not seen when nuclear proteins prepared from hormone-independent cells (MB468 and MB231) treated either with or without RA were used. Thus, a lack of βRARE binding activity may be responsible for the loss of βRARE transcriptional activation in the hormone-independent cells.

Recovery of RA sensitivity in hormone-independent breast cancer cells by RARβ expression.

The above data suggest that induction of RARβ by RA may be responsible for the RA-induced growth inhibition in hormone-dependent breast cancer cells and that the loss of RA sensitivity in hormone-independent breast cancer cells may be due to a lack or low levels of RARβ in these cells. To directly test this, cDNA for the RARβ gene was cloned into pRc/CMV vector so that the expression of the RARβ gene is under the control of cytomegalovirus (CMV) promoter. The vector also contains a neomycin resistance gene that allows transfected cells grow in the presence of G418. pRc/CMV-RARβ was transfected into hormone-independent breast cancer cells (MB231). Six neomycin resistance MB231 clones were selected that carried the exogenous RARβ gene as revealed by Southern blot analysis (data not shown). Among these clones, MB231/RARβ2 and MB231/RARβ3 expressed exogenous RARβ gene transcripts (figure 4a) as judged by its smaller size than the endogenous RARβ transcript observed in RA-treated ZR-75-1 cells (data not shown). The rest of the clones and cells transfected with pRc/CMV empty vector did not show any RARβ transcript.

To determine the effect of the introduced RARβ, the growth of MB231/RARβ2, MB231/RARβ3, MB231/RARβ9, and cells transfected with empty vector was measured in the presence or absence of either all-trans RA or 9-cis RA by MTT assay (figure 4b). In the presence of RA, we observed a strong growth inhibition of RARβ-transfected cells. Treatment of 10^{-6} M RA resulted in about 50% inhibition of MB231/RARβ2 cell growth.

Under the same condition, about 40% of inhibition was seen in MB231/RARβ3 cells. In contrast, MB231 cells transfected with empty vector or parental MB231 cells did not show any response to RAs. The fact that cells (MB231/RARβ9) transfected with pRc/CMV-RARβ vector but failed to express RARβ (figure 4a) did not show any response to RAs (figure 4b) suggests that the growth inhibitory effect of RAs is mediated by RARβ product. To further characterize the effect of transfected RARβ gene, the RARβ–transfected cells were analyzed for their anchorage-independent growth in soft agar. As shown in figure 5, the growth of transfectant cells that expressed RARβ (MB231/RARβ2 and MB231/RARβ3) in soft agar was dramatically inhibited by RA whereas the growth of parental MB231 cells was not affected. Together, these data demonstrate that the expression of RARβ can restore RA sensitivity in hormone-independent breast cancer cells.

Induction of RARβ by RARα is responsible for recovery of RA sensitivity in hormone-independent breast cancer cells.

The expression of RARα is relatively low in some of the hormone-independent cell lines, such as MB231 and MB-468 (figure 2b). This has been previously suggested to account for RA resistance in hormone-independent cells (70, 71). We then investigated whether the expression of RARα could restore RA sensitivity in hormone-independent cells. RARα cDNA was cloned into pRc/CMV vector and the resulting expression plasmid was stably transfected into MB231 cells. Two clones (MB231/RARα1 and MB231/RARα2) that expressed introduced RARα gene (data not shown) showed a strong RA growth inhibitory effect in a concentration-dependent manner while it had little effect on the growth of parental MB231 cells (figure 6a). These data are consistent with previous observation (70). Since RARα is known to activate βRARE (15, 30, 72). We, therefore, investigated whether expression of the introduced RARα could result in induction of endogenous RARβ. As shown in figure 6b, the expression of the endogenous RARβ was significantly enhanced in several different clones that expressed exogenous RARα when these cells

were treated with 10^{-6} M RA. Thus, activation of RARα can result in induction of RARβ in breast cancer cells. Furthermore, Ro 41-5253 (2), a RARα selective antagonist, strongly inhibited the induction of RARβ by RA in T-47D and ZR-75-1 cells (figure 6c). Together, the growth inhibitory effect of RARα is likely due to its induction of the endogenous RARβ through activation of the βRARE.

RARβ is essential for RA-induced growth inhibition in hormone-dependent cells.

To investigate whether the induction of RARβ by RA can mediate the RA-induced growth inhibition in hormone-dependent cells, we used a RARβ selective antagonist (LE135) (19, 47) to suppress RARβ activity in the cells. This retinoid can specifically inhibit RARβ- but not RARα- or RARγ-mediated activation of target genes (47). As shown in figure 7a, a concentration dependent reduction in RA-induced growth inhibition was observed in both ZR-75-1 and T-47D cells when LE135 was added to cells together with 10^{-7} M RA. In the presence of 10^{-6} M LE135, RA-induced growth inhibition in ZR-75-1 cells was reduced from about 50% to 20%. Similar degree of effect was seen in T-47D cells. These data suggest that activation of RARβ is mainly responsible for RA-induced growth inhibition in hormone-dependent breast cancer cells. To further support this conclusion, we stably transfected a RARβ anti-sense cDNA that cloned into pRc/CMV into ZR-75-1 cells. Expression of anti-sense mRNA is known to reduce its target mRNA level by their hybridization which results in degradation of the double stranded RNA (8). As shown in figure 7b, clone A-RARβ5 did not show detectable expression of RARβ anti-sense RNA. The expression of the endogenous RARβ in this clone is highly induced by RA similar to that of parental cells as determined by Northern blot analysis (figure 7b and data not shown). Clone A-RARβ10 expressed RARβ anti-sense RNA and failed to express endogenous RARβ under RA treatment (figure 7b). Therefore, expression of RARβ anti-sense RNA significantly inhibited RA-induced expression of the endogenous RARβ.

Furthermore, when the growth inhibitory effect of RA was examined in two clones which expressed RARβ anti-sense RNA (A-RARβ10 and A-RARβ25) (figure 7b and data not shown), we observed a reduced RA sensitivity to RA-induced growth inhibition in these clones (figure 7c). In contrast, the growth of ZR-75-1 cells which were stably transfected with the same vector but failed to express RARβ anti-sense RNA (A-RARβ5) remained strongly inhibitable by RA (figure 7c). These results, therefore, demonstrate that expression of RARβ is essential for RA-induced growth inhibition in hormone-dependent breast cancer cells.

RA-activated RARβ promote apoptosis in breast cancer cell.

When RARβ was expressed in MB231 cells, we noticed a morphology change of the cells. MB231 cells, when seeded at low density, were elongated (figure 8a). However, MB231 cells that expressed RARβ were relatively round and became shrunken. Such a morphology change was observed even in the absence of RA, probably due to constitutive high level of RARβ expression and the presence of residual amount of retinoids in the serum. The ability of the cells that expressed RARβ to survive on culture dishes was quite different from wild-type cells. After continuation of culture at low density, a large portion of cells eventually died, particularly in the presence of RA as assayed by trypan blue dye exclusion (data not shown). To investigate whether the loss of survivability of the cells is due to apoptosis, we examined nuclear morphology of MB231 cells that expressed RARβ. When the nuclei of these cells were stained by PI and examined by confocal fluorescence microscopy, we found that many of the RA-treated MB231/RARβ3 cells were smaller and contained condensed and fragmented nuclei with brightly staining chromatin, i.e., morphological changes typical of apoptosis (75) (figure 8b). RA caused similar nucleus morphological alterations in the RA-sensitive lines ZR-75-1, MCF-7 and T-47D (figure 8b) but not in the RA-resistant lines MB231, MB-468 and BT-20 (data not shown).

To further study RA-induced apoptosis in breast cancer cells, we carried Terminal Deoxynucleotidyl Transferase Assay (TUNEL) with flow cytometric analysis to study DNA fragmentation. As shown in figure 8c, stable expression of RARβ in MB231 cells (MB231/RARβ3) resulted in significant amounts of TdT-labeled cells when they were treated with 10^{-6} M all-trans RA for 24 hr. While treatment of MB231 cells with all-trans RA did not show clear increase of TdT-labeled cells. In ZR-75-1 cells, a marked increase in TdT-labeled cells was observed in response to all-trans RA. However, the TdT-labeling was significantly inhibited when RARβ anti-sense RNA was expressed (A-RARβ10). Similar results were obtained by another assay (figure 8d) which is based on the sandwich-enzyme-immunoassay-principle to determine cytoplasmic histone-associated DNA fragments in apoptotic cells. In addition, this study revealed that DNA fragmentation in ZR-75-1 and MB231/RARβ3 occurred as early as 12 hrs after exposing cells to RA. These results clearly demonstrate that RA can induce apoptosis in breast cancer cells and that RA-induced apoptosis is mediated by RARβ in the cells.

Discussion

Retinoids are effective growth inhibitors of breast cancer cells. However, the inhibition of the growth is often observed in hormone-dependent but not in hormone-independent breast cancer cells (21, 76). In this study, we have investigated the mechanism by which the differential growth inhibitory effect of RA is mediated. Several lines of evidence indicate that expression of RARβ gene is critical for the effect. First, RA-induced RARβ gene expression in breast cancer cell lines correlated with the RA-induced growth inhibition in these cell lines (figure 2). Secondly, hormone-independent cells that were devoid of RARβ gained RA-sensitivity when RARβ was expressed in the cells (figures 4 and 5). Thirdly, in hormone-dependent cells, RA sensitivity was inhibited when their endogenous RARβ activity was suppressed by a RARβ-selective antagonist and the expression of RARβ anti-

sense RNA in the cells (figure 7). Finally, activation of RARβ was able to promote apoptosis (figure 8).

The growth inhibitory effect of retinoids is presumably mediated by the retinoid receptors. In this study we have examined the contribution of each retinoid receptor to the RA-induced growth inhibition. By using receptor selective retinoids, we first shown that activation of RXRs is not involved (figure 1). Retinoid that activates RXR and induces RXR homodimer formation did not show clear effect on the growth of two different hormone-dependent breast cancer cell lines (ZR-75-1 and T-47D) while retinoids that activate RARs were as effective as all-trans RA. These data are consistent with results obtained by a previous study that used different RXR-selective retinoids in MCF-7 and other breast cancer cell lines (70). All-trans RA and 9-cis RA displayed a similar growth inhibition pattern in several breast cancer cell lines (figure 2) and in MCF-7 cells (67) although 9-cis RA may be slightly more active. These results demonstrate that selective activation of RXR homodimer pathway does not contribute substantially to the RA-induced growth inhibition in breast cancer cells and suggest that activation of RAR pathway may be critical. Previously, several studies were carried out to elucidate the role of RARs in the differential growth inhibitory effect of RA in hormone-dependent and -independent breast cancer cell lines (66, 76). These studies and the present study (figure 2) showed comparable expression levels of RARγ mRNA in all the cell lines regardless of the ER status and no marked expression level changes under RA treatment indicating that RARγ is unlikely involved. However, RARγ may function to mediate the synergistic growth inhibitory effect of RA and interferon on breast cancer cells (79). Roman et al. (66) found that RARα was expressed in all cell lines with slight higher levels in hormone-dependent cell lines than in independent lines. In another study (76), RARα transcript was found in the hormone-dependent lines but was almost absent in -independent lines except BT-20. In our study, two RAR transcripts were observed in all cell lines investigated. However, they were less

abundance in MB231 and MB468 cell lines (figure 2) but was highly expressed in another hormone-independent line (BT-20). Although these studies suggest that RARα may be involved, the slight variations in the expression levels of RARα (66 and this study) can not satisfactorily explain the dramatic difference in the sensitivities of different breast cancer cell lines to RA. So far, the expression of RARβ has been investigated by several studies (66, 73, 76). However, results obtained are inconsistent and sometimes contradict each other. van der Burg et al (76) observed high levels of RARβ in two of the three hormone-dependent lines (ZR-75-1 and T-47D) but not in independent lines except Hs578T. In contrast, RARβ was expressed in all independent cell lines analyzed by Roman et al (66) while it was not detected in dependent lines including T-47D and MCF-7 or expressed at a low level in other dependent lines. In another study (73), RARβ transcript in MCF-7 and ZR-75-1 cells could only be detected when poly A RNA was used. Thus, the role of RARβ could not be established. These different results obtained may be due to variability of cell lines used, and more likely due to different culture conditions used. It is well known that the expression of RARβ is very sensitive to RA regulation due to the presence of a RARE (βRARE) in its promoter (15, 30, 72) in many different cell types, including breast cells (73). Thus trace amounts of retinoids in culture media may result in very different expression levels of the gene. To clearly establish the role of RARβ, we have examined the expression of RARβ in several hormone-dependent and -independent cell lines either in the presence or in the absence of RA (figure 2). In the absence of RA, all of these cell lines did not exhibit detectable RARβ transcript, similar with results previously reported (73). However, in the presence of RA, the expression of RARβ was strongly enhanced in hormone-dependent cell lines (ZR-75-1, T-47D and MCF-7) but not in independent cell lines (MB231, BT-20 and MB468). Thus, RA-induced RARβ expression correlates with RA-induced growth inhibition in these cells, suggesting that RARβ may be directly involved in the differential growth inhibitory effect of RA in breast cancer cell lines.

The role of RARβ in mediating RA-induced growth inhibition is supported by our stable transfection study in hormone-independent cells (MB231) (figure 4). The growth of MB231 cells that are devoid of RARβ did not show response to RA (figure 2). However, when RARβ gene was introduced and expressed in the cells, the inhibitory effects of RA on the anchorage-dependent and -independent growth of the cells was observed (figures 4 and 5). The restoring of RA responsiveness is specific since it was observed in several individual transfectants. In addition, cells transfected with empty vector or transfected with pRc/CMV-RARβ but failed to express RARβ did not exhibit RA response. These data provide a direct evidence for the function of RARβ in RA-induced growth inhibition in breast cancer cells. This is further supported by our studies in hormone-dependent cells. First, we used a RARβ-selective antagonist (LE135) (47) to test whether it could abolish RA-induced growth inhibition in these cells. LE135 is an effective RARβ-selective antagonist (47). It binds RARβ with a high affinity ($Ki=4.3 \times 10^{-8}$ M), which is about two-order stronger than to RARα. It can efficiently prevent RA-induced RARβ activity in transient transfection assay, while it has no effect on RARα and RARγ activity (47). When we used LE135 together with all-trans RA, we found that it could significantly prevent RA-induced growth inhibition on ZR-75-1 and T-47D cells (figure 7). Furthermore, we investigated whether expression of RARβ anti-sense RNA could abolish or decrease RA-induced growth inhibition in the cells. Two clones obtained that expressed RARβ anti-sense RNA showed a strong reduced RA sensitivity (figure 7). Together, these data clearly demonstrate that RARβ can mediate the RA-induced growth inhibition in breast cancer cells. This conclusion is supported by a recent study showing that senescence of normal human mammary epithelial cells resulted in increased RARβ mRNA expression (73).

Previous studies (66, 71, 76) and this studied show that RARα transcripts are not expressed or expressed with relative low levels in certain hormone-independent cell lines.

These data suggested that RARα might account for the growth inhibition of RA. How RARα is underexpressed in certain hormone-independent breast cancer cell lines and how it is involved in mediating RA-induced growth inhibition remain to be seen. In this study, we did not observe any enhancement of RARα transcript in cells that expressed introduced RARβ (data not shown), indicating that the recovery of growth inhibitory effect of RA observed in these cells is unlikely mediated by RARα. Similar to a previous observation (70), we found that RARα could restore RA sensitivity in the cells when it was highly expressed in MB231 cells (figure 6). However, when we analyzed the expression of endogenous RARβ in cells that expressed introduced RARα, we found that it was significantly enhanced in the presence of RA (figure 6c). In our transient transfection assay, we observed that cotransfection of RARα could enhance βRARE activity in hormone-independent cells (our unpublished results). In addition, Sheikh et al (70) reported that the βRARE activity was higher in clones that stably expressed RARα than their mock-transfected counterpart. Together, these observations suggest that the effect of RARα may be in part due to its activation of endogenous RARβ, most likely through activation of the βRARE. However, RARα alone may not be sufficient to render breast cancer cells RA responsiveness as seen in BT20 cells that express a relative high level of RARα transcript (figure 2a) but nevertheless are RA resistance (figure 2d). It is likely, therefore, that other nuclear protein(s) may participate in the regulation of RA responsiveness in breast cancer cells.

The growth inhibitory effect of RA appears to be depended on ER status. Whether this is due to a coincidence resulted from progression of tumor or whether estrogen somehow influences RA activities remains to be elucidated. In our stable transfectants that expressed RARβ, we did not observe any change of ER expression levels (data not shown), indicating that ER activities are not involved in RARβ-mediated growth inhibition in breast cancer cells. Recently, we have observed that RA can inhibit ER transcriptional activity in

breast cancer cells (42). Rubin et al (67) also reported that 9-cis RA could down-regulate ER activity in MCF-7 cells. These data demonstrate a possible cross-talk between retinoid and estrogen signaling in breast cancer cells, that may influence RA-induced growth inhibition in breast cancer cells.

The loss of RA response in inducing RARβ gene expression in the hormone-independent human breast cancer cell lines indicates that RA response is impaired in these cell lines. This is further demonstrated by our transfection and DNA binding assays, showing a diminished βRARE binding and transcriptional activation (figure 2). The ability of RARα to activate βRARE in hormone-independent cells raises a question of why endogenous RARα in hormone-independent cells can not activate the βRARE. So far we do not understand how endogenous βRARE is activated. In our previous report (87), we have demonstrated that the loss of RA up-regulation of RARβ gene expression may be due to multiple defects. We have recently observed that a nuclear protein other than RAR and RXR may function as retinoid receptor co-activator on the βRARE and may be essential for its activation. The nuclear protein is expressed in a relative low level in hormone-independent breast cancer cells than in hormone-dependent cells. This may explain the loss of βRARE DNA binding and transactivation activity in hormone-independent breast cancer cells (figure 3) even though RARs (figure 2) and RXRα and RXRβ (data not shown) are expressed in the cells. However, overexpression of RARα appears to be able to overcome low expression of the nuclear protein in hormone-independent breast cancer cells and confer their RA responsiveness.

In this study, we have present evidence that RARs can mediate the RA-induced growth inhibition in breast cancer cells. However, the observations (69, 74) that retinoids that did not bind to any subtype of RAR are an effective growth inhibitors suggest that different mechanism of action or target other than RARs may mediate the retinoid-induced growth

inhibition. The mechanisms by which RARs negatively regulate growth are largely unknown. A possible mechanism is their inhibitory effect on the transcriptional activity of proto-oncogenes cJun and cFos, the components of AP-1, which are commonly associated with cell proliferation (68, 80). RARβ, in response to RA, may repress the activity of the AP-1 and thereby contributes to its growth inhibitory effect. In this study, we provide evidence that induction of apoptosis by RARβ may represent another important mechanism by which RARβ exerts its growth inhibitory function. Apoptosis, as a distinct form of cell death, is an important process that can lead to tumor regression. Suppression of apoptosis results in abnormal cell survival and malignant growth. Apoptosis of lymphoblasts induced by steroid hormones, such as glucocorticoid, has been suggested to account for the antileukemic activity of these agents (75). RA was also reported to induce cell death in hematopoietic cells (60), and in breast cancer cells (63, 69). The observations (17, 56) that the expression of RARβ in the developing mouse limb is highly restricted to the mesenchyme of the interdigital regions destined to undergo apoptosis (39) suggest that RARβ could be associated with programmed cell death. Here we provide direct evidences that RARβ is able to mediate RA-induced apoptosis of breast cancer cells. RA-induced apoptosis was observed in ZR-75-1 cells that highly expressed RARβ gene in the presence of RA. Inhibition of RARβ activity by the expression of RARβ anti-sense RNA reduced the number of apoptotic cells (figure 8d). In contrast, RA-induced apoptosis was observed in hormone-independent cells only when RARβ was introduced and expressed in the cells (figure 8d). Although RA-promoted apoptosis may contribute to RA-induced growth inhibition, apoptosis alone may not be sufficient to account for the strong growth inhibitory effect of RA observed in breast cancer cells. For example, induction of apoptosis by RA in ZR-75-1 cells was much higher than in T-47D cells (data not shown) although a similar degree of RA-induced growth inhibition was observed in these two cell lines (figure 2D). Thus, other mechanism, such as anti-AP-1 effect, may be also employed.

RARβ is expressed in normal breast tissue (73). Furthermore, RARβ mRNA is induced by RA in normal human breast cell lines (73). In our study, RA-induced RARβ expression was only observed in hormone-dependent breast cancer cell lines but not in independent cell lines. Since hormone-independent breast cancer cells are usually considered to represent those at a late stage of breast tumor progression, our observation suggests that the loss of the RARβ gene expression may have important pathogenic consequence during the development of human breast cancer. The association between RARβ and cancer development was originally implicated in the finding that RARβ might be involved in the development of human liver cancer (14). Recently, more and more evidence are emerging showing that the loss of the RARβ gene expression may be also associated in the development of other types of cancers (23, 31, 33, 62, 87). In lung and breast cancer, a deletion of the short arm of chromosome 3p, a region that maps close to the RARβ gene, occurs with high frequency (16, 61, 81). Abnormal low levels of the RARβ gene expression was observed in many human lung cancer cell lines and other cancer cell lines (23, 31, 33, 62, 87), and was suggested to contribute to neoplastic progression of human oral squamous cell carcinoma cell lines (33) and the tumorigenicity of papillomavirus 18 transformed Hela cells (3). Furthermore, RARβ has been shown to function as a tumor suppressor gene in epidermoid lung carcinoma cells (32). These observations, therefore, suggest that a low expression level of RARβ gene may be an important contributing factor for cancer development. The mechanism by which the loss of the RARβ gene expression contributes to cancer development is unclear. Induction of RARβ by RA presumably plays a critical role in amplifying the RA responses. Therefore, the failure of RA to upregulate RARβ gene expression suggests that cancer cells may have lost most, if not all, their RAR activities. The association between altered RAR transcriptional activities and carcinogenesis was clearly demonstrated in APL cells where abnormal RARα fusion transcripts were produced by the characteristic chromosomal translocation (13, 37). RARβ, in response to RA, can inhibit the effect of tumor promoter

TPA and the transcriptional activity of proto-oncogenes cJun and cFos (68, 80), and induce cellular apoptosis, a process that can lead to tumor regression. Loss of RARβ activities could remove such negative control mechanisms, resulting uncontrolled cell proliferation and therefore enhances the transformed phenotype of cells.

In conclusion, our results demonstrate that the loss of RARβ gene expression and regulation by RA is a common feature associated with hormone-independent breast cancer cells and may be one of the major factors responsible for the diminished retinoid sensitivity during the progression of breast tumor. Thus, the expression level of the RARβ gene and its response to RA could serve as diagnosis factors for these cancers and could be also used to determine whether patients with breast cancer will respond to RA treatment. Our results also demonstrate that RARβ mediates the growth inhibitory effect of RA in part by inducing cell apoptosis, which when lost may contribute to cancer development. The observation that introduction of RARβ gene into RARβ negative cancer cells can restore RA responsiveness provides valuable directions for developing new strategies in the treatment of human breast cancer. Further analysis of the RA response of various cancer cells introduced with the RARβ gene will significantly enhance our understanding of the relationship between the abnormal expression of the RARβ gene and diminished sensitivity of cancer cells to RA therapy.

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Note added in proof

As we are preparing this manuscript, there is a paper published in the September issue of Cell Growth & Differentiation (Seewaldt, V. L., Johnson, B.S., Parker, M.B., Collins, S.J., and Swisshelm, K. 1995. Expression of retinoic acid receptor β mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. 6: 1077-1088). In that paper, the authors reported that RARβ can mediate growth arrest and induce apoptosis in human breast cancer cells which support our discovery in this manuscript.

Figure Legends

Figure 1. Activation of RARs but not RXRs is required for RA-induced growth inhibition in breast cancer cell lines. Effect of RAR/RXR heterodimer [Ch55 and (all-E)-**UAB8**] and RXR homodimer [(9Z)-**UAB8**] specific activators on the growth of hormone-dependent breast cancer cells (ZR-75-1 and T-47D). The effect of all-trans RA was used for comparison. Cells were seeded at 1,000 cells per well and treated with 10^{-7} M retinoids for 10 days. The results were expressed as a percentage of absorbance at 550 nm of MTT-derived formazan developed by cells treated with control solvent. Filled circle represents all-trans RA treatment and open diamond indicates 9-cis RA treatment. All data shown are representative of three independent experiments.

Figure 2. Expression of retinoid receptors and growth inhibitory effect of RA in human breast cancer cells. Expression of RARγ (**a**), α (**b**), and β (**c**) in hormone-dependent and -independent human breast cancer cell lines. The expression of RAR genes was determined by Northern blot analysis using total RNA (about 30 μg) prepared from different breast cancer cell lines. To determine the effect of RA, cells were treated with 10^{-6} M RA for 36 hours before RNA preparation. **d.** Effect of all-trans RA and 9-cis RA on the growth of hormone-dependent and -independent breast cancer cells. 2,000 cells per well were seeded and treated with various concentrations of RAs for 7 days. Growth inhibition was performed as described in figure 1.

Figure 3. Transcriptional activity and binding of βRARE in human breast cancer cell lines. a. Transcriptional Activation of βRARE in hormone-dependent and -independent human breast cancer cell lines. Transient transfection assays were used to determine transcriptional activation of βRARE in various human breast cancer cell lines. **b.** βRARE binding of nuclear proteins prepared from hormone-dependent and -independent human breast cancer cell lines. Arrow indicates the specific binding complex present in breast cancer cells.

Figure 4. Stable expression of RARβ genes restores RA sensitivity in hormone-independent RA-resistant breast cancer cells. a. Northern blot analysis of stable transfectants for RARβ gene expression. Total RNA (30 μg) was prepared from

different stable transfectants and analyzed for RARβ gene expression. **b.** Inhibition of anchorage-dependent growth of MB231 cells by stable expression of RARβ. The growth of the RARβ stable transfectants (MB231/RARβ2, MB231/RARβ3, and MB231/RARβ9), parental MB231, and MB231 cells transfected with empty vector (MB231/vector) in the absence or presence of RA was determined as described in figure 1.

Figure 5. Inhibition of anchorage-independent growth of MB231 cells by RARβ gene expression. a. Visualization of colonies formed by parental MB231, MB231/RARβ2 and MB231/RARβ3 cells. **b.** Quantitation of colonies formed by parental MB231, MB231/RARβ2 and MB231/RARβ3 cells. Colonies formed by MB231/RARβ2, MB231/RARβ3 and parental MB231 cells in the presence or absence of all-trans RA were scored and expressed as percentage of colonies formed by cells treated with control solvent.

Figure 6. Inhibition of anchorage-dependent growth of MB231 cells by stable expression of RARα. a. The growth of RARα stable transfectants (MB231/RARα1 and MB231/RARα2) and parental MB231 cells was analyzed in the presence of various concentrations of all-trans RA as described in figure 1. **b.** Expression of endogenous RARβ gene in RARα stable transfectants. Northern blot was used to analyze the expression of endogenous RARβ gene in MB231 cells that stably expressed RARα in the presence of 10^{-6} M all-trans RA. For comparison, the expression of RARβ in ZR-75-1 cells treated with all-trans RA was shown. The expression of L32 gene was used as control. **c.** Inhibition of RA-induced RARβ gene expression by RARα selective antagonist. The expression of RARβ gene in ZR-75-1 and T-47D cells in the presence of 10^{-7} M all-trans RA together with or without 10^{-6} M RARα selective antagonist (Ro 41-5253) was analyzed by Northern blot as described in figure 2.

Figure 7. Inhibition of RARβ activity decreases RA sensitivity in hormone-dependent breast cancer cells. a. RARβ selective antagonist decreases the growth inhibitory effect of RA in ZR-75-1 and T-47D cells. ZR-75-1 and T-47D cells were treated with or without 10^{-7} M all-trans RA in the presence or absence of RARβ selective antagonist (LE135 Ki for RARα: 1.5×10^{-6} M; Ki for RARβ: 4.3×10^{-8} M) for 10 days and the numbers of the cells were analyzed by MTT assay as described in figure 1. **b.** Expression of RARβ in ZR-75-1 cells transfected with RARβ anti-sense cDNA. The expression of

endogenous RARβ in two stable clones (A-RARβ5 and A-RARβ10) was determined by Northern blot. A-RARβ10 expressed introduced RARβ anti-sense RNA while no clear expression was seen in A-RARβ5. **c.** Expression of RARβ anti-sense RNA decreases the RA sensitivity in hormone-dependent breast cancer cells. The effect of all-trans RA on the growth of ZR-75-1 cells and ZR-75-1 cells that stably expressed RARβ anti-sense RNA (A-RARβ10 and A-RARβ25) was analyzed by MTT assay as described in figure 1.

Figure 8. RARβ promotes cell apoptosis. **a.** Morphology change of MB231 cells by RARβ gene expression. **b.** Morphological analysis of apoptotic breast cancer cells stained with PI after RA treatment. A, B, C and D represent breast cancer cell line MB231/RARβ3, ZR-75-1, T-47D and MCF-7, respectively. MB231/RARβ3 and ZR-75-1 cells were treated with 10^{-6} M RA for 48 hours. T-47D and MCF-7 cells were treated with 10^{-6} M RA for 72 hours. Arrows indicate the apoptotic nuclei. **c.** TUNEL assays of parental and RARβ transfected MB231 (MB231/RARβ3) cells, and parental and antisense RARβ transfected ZR-75-1 (ZR-75-1/A-RARβ10) cells after 24 hours of RA treatment. **d.** ELISA analysis of DNA fragmentation in apoptotic breast cancer cells.

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figure 1

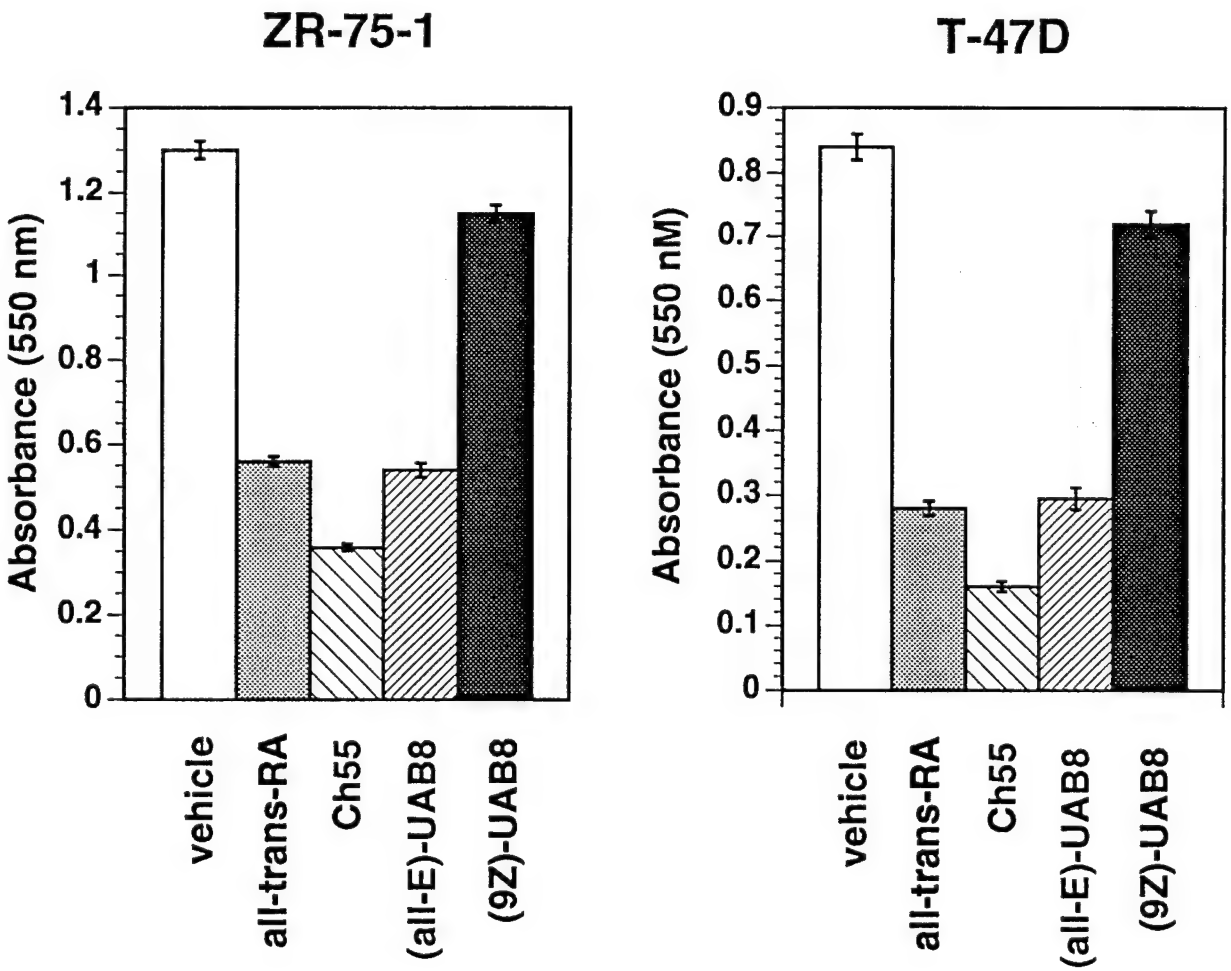

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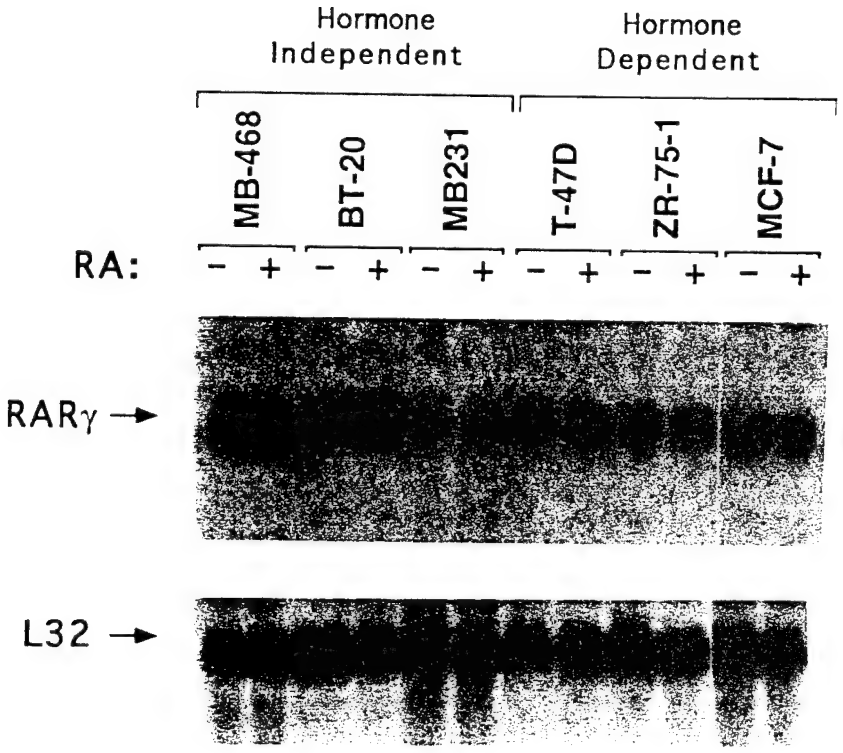
figure 2a



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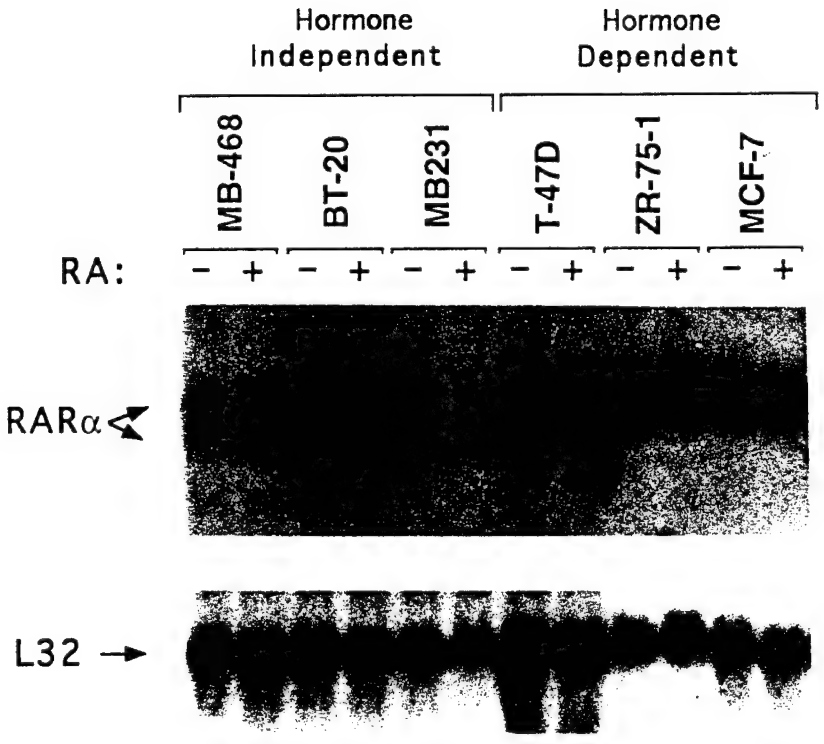

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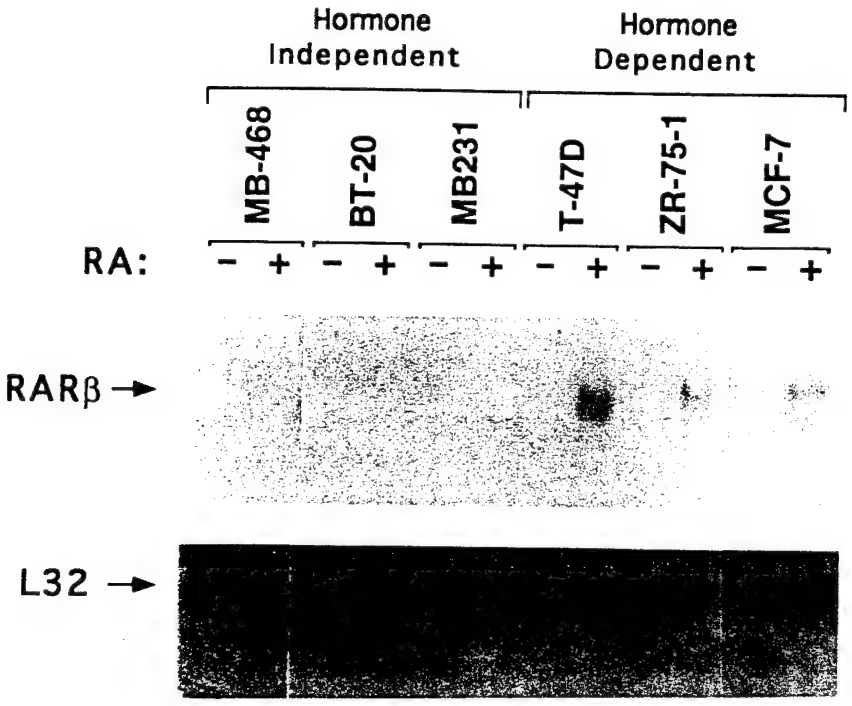

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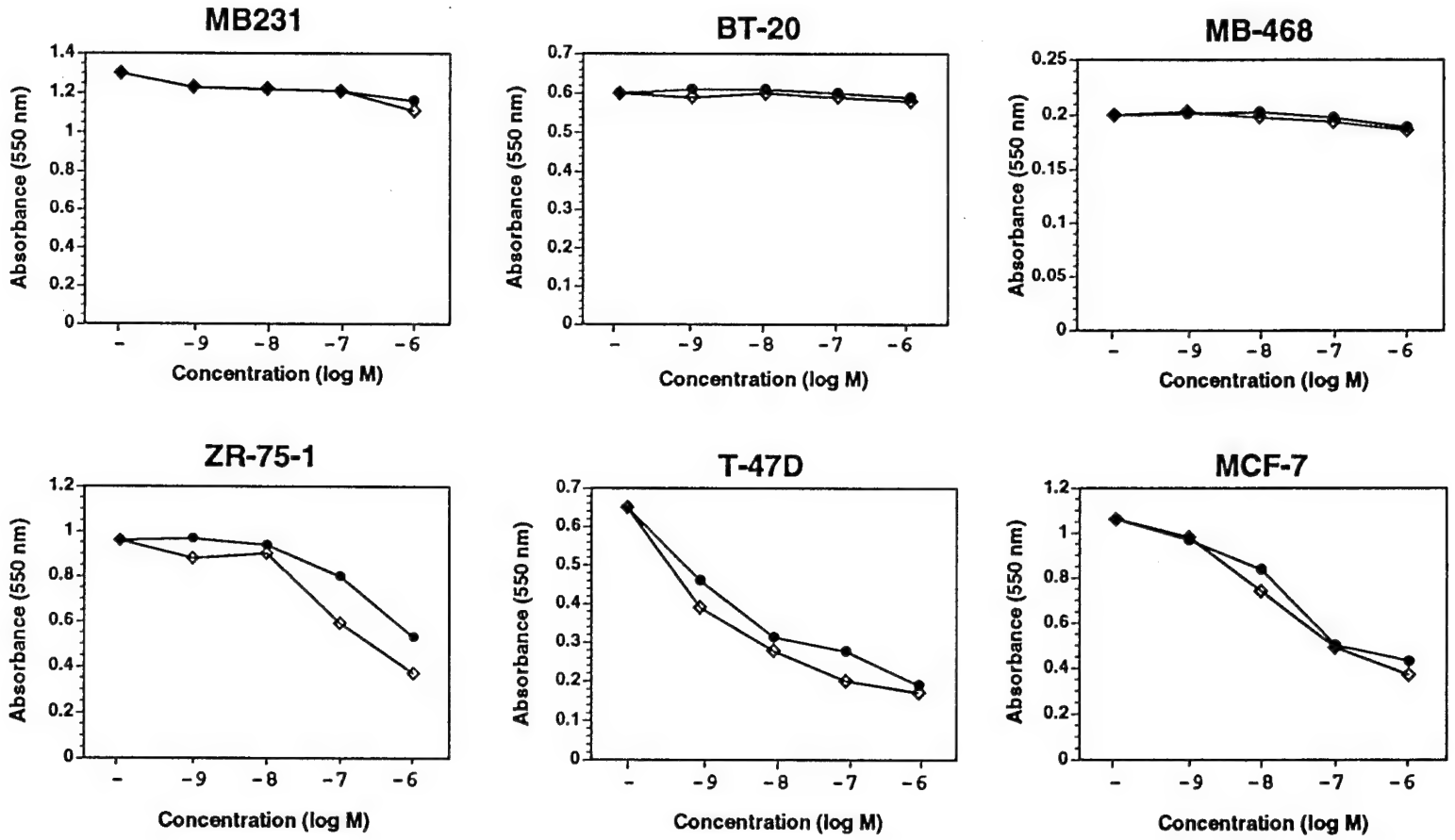

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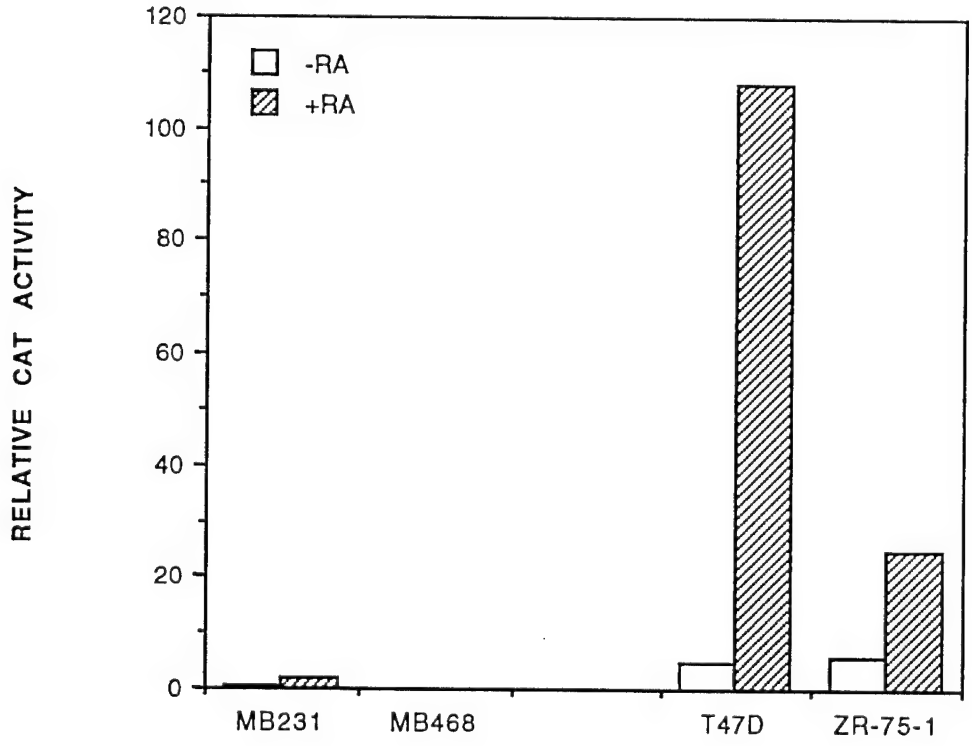

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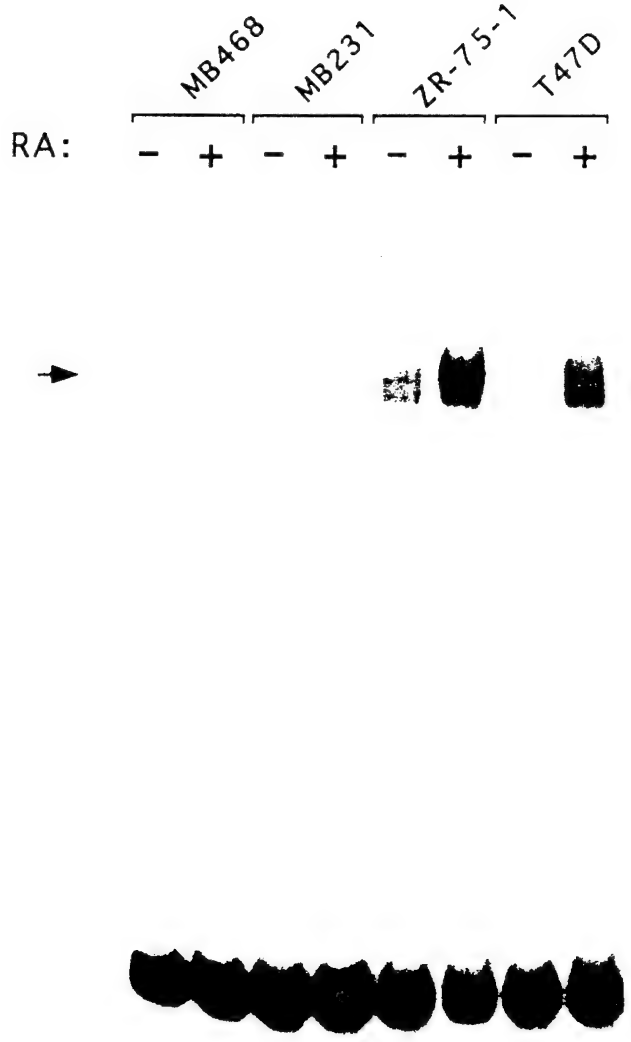

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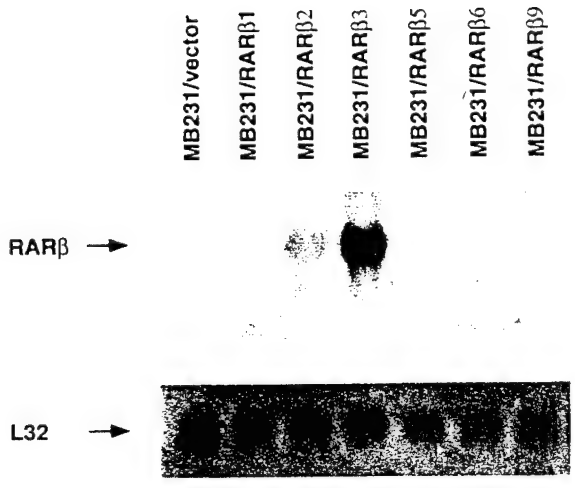

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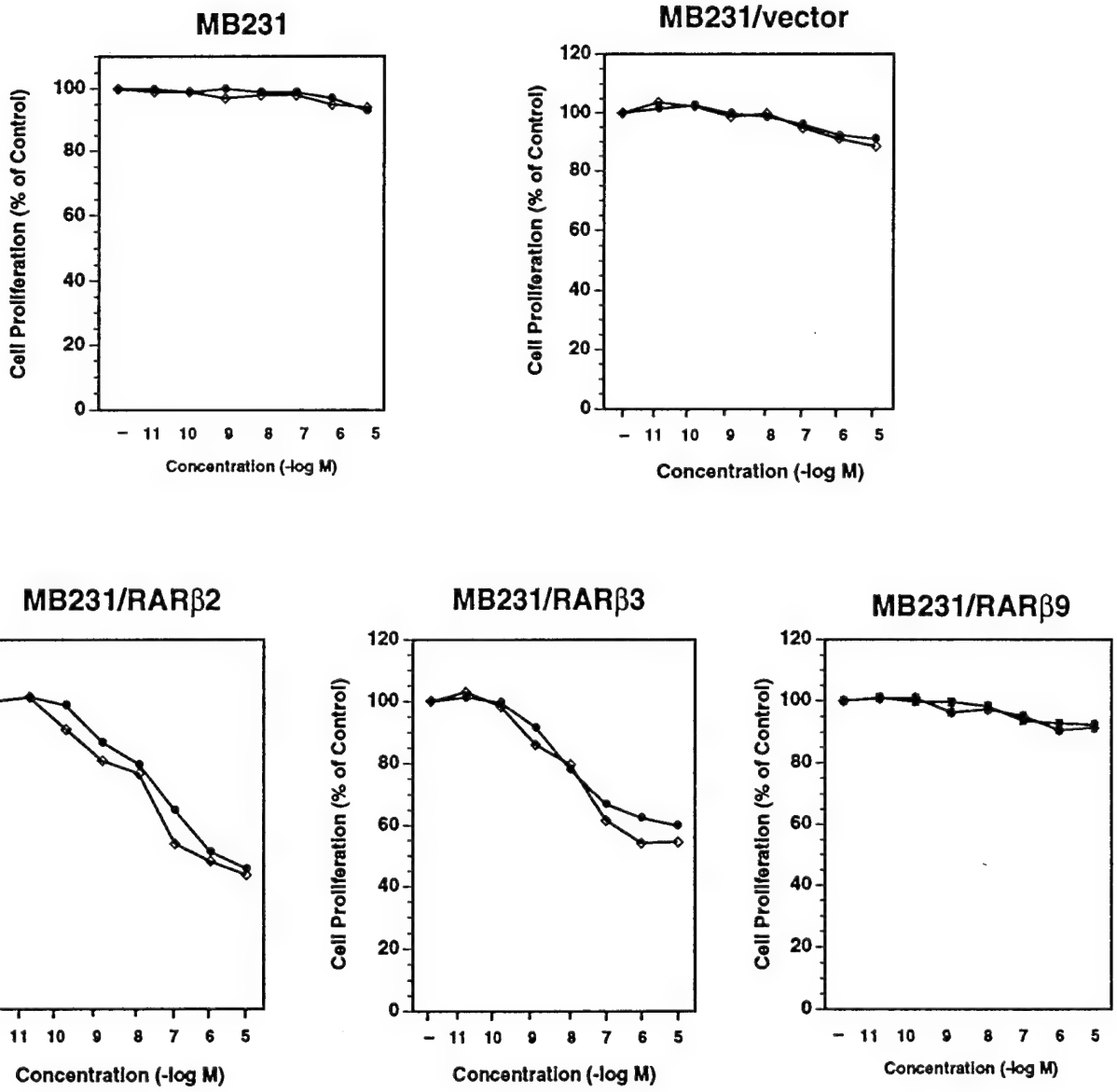

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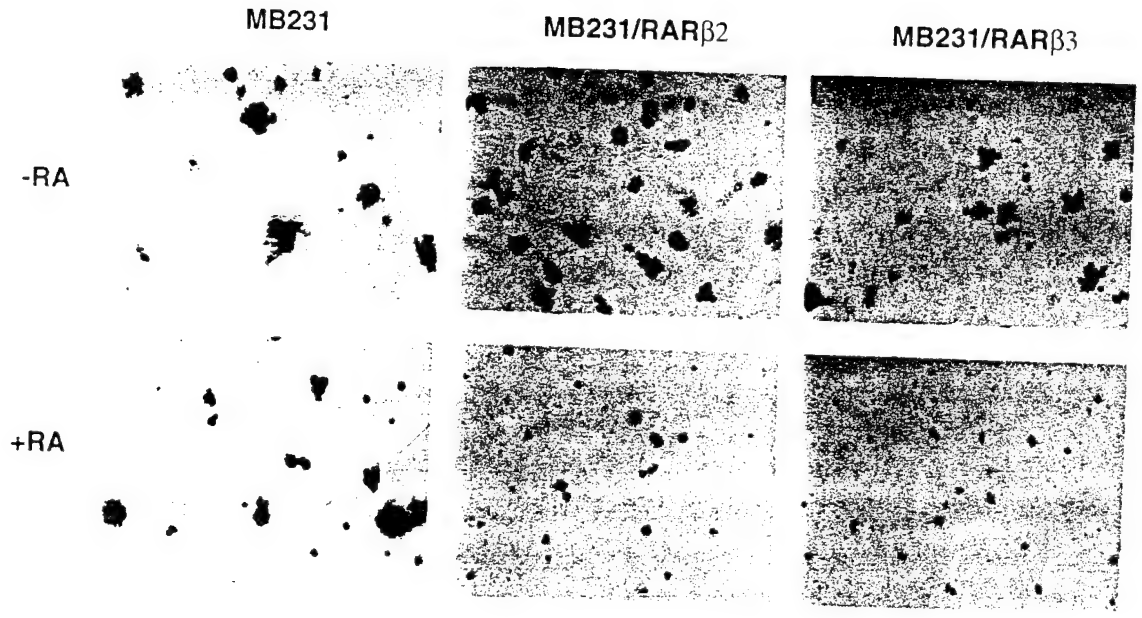

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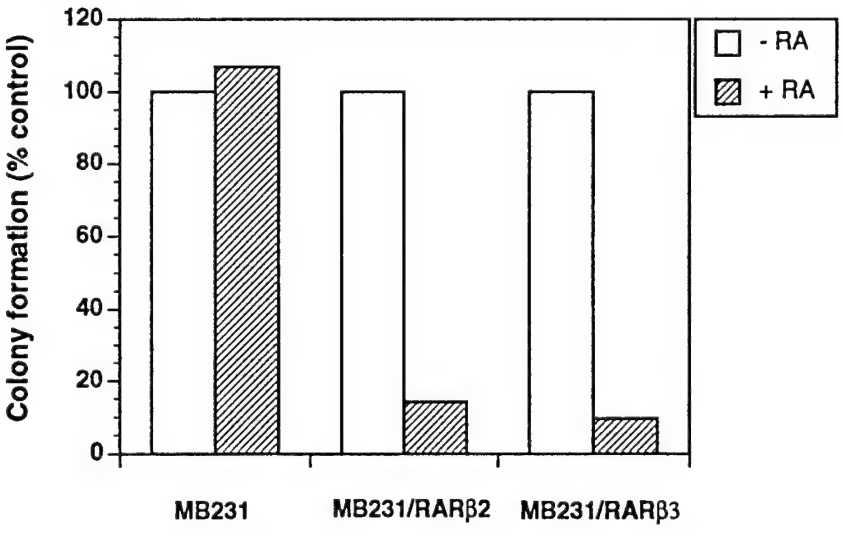

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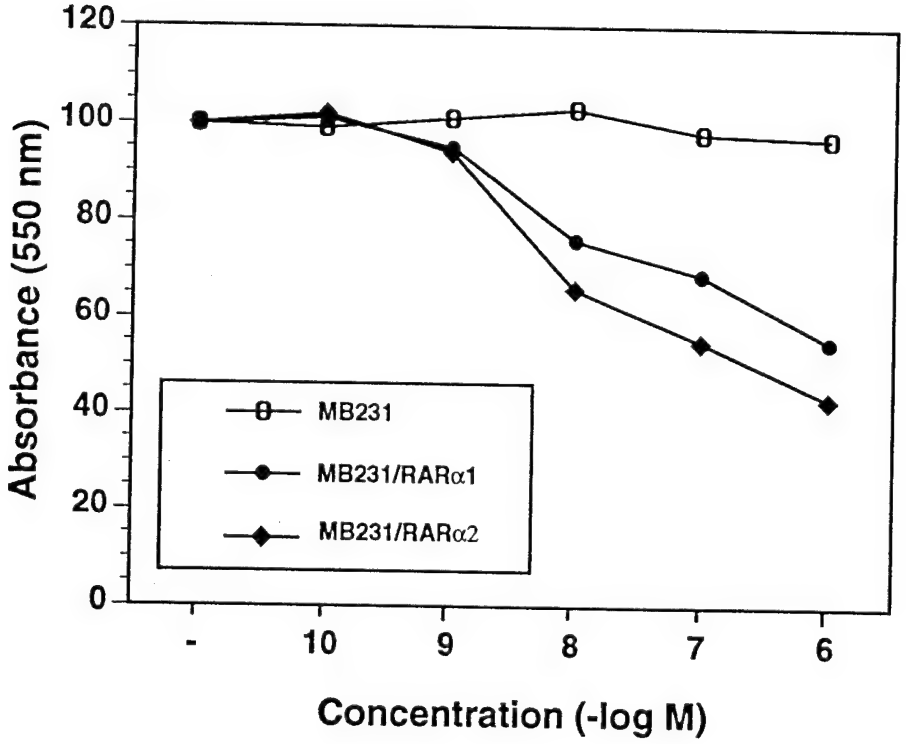

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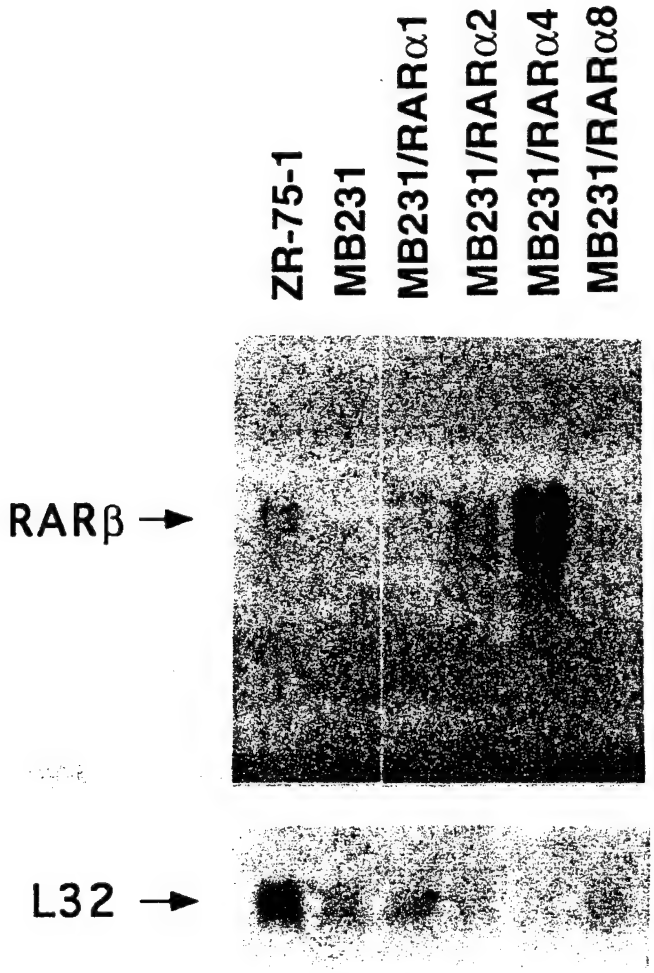

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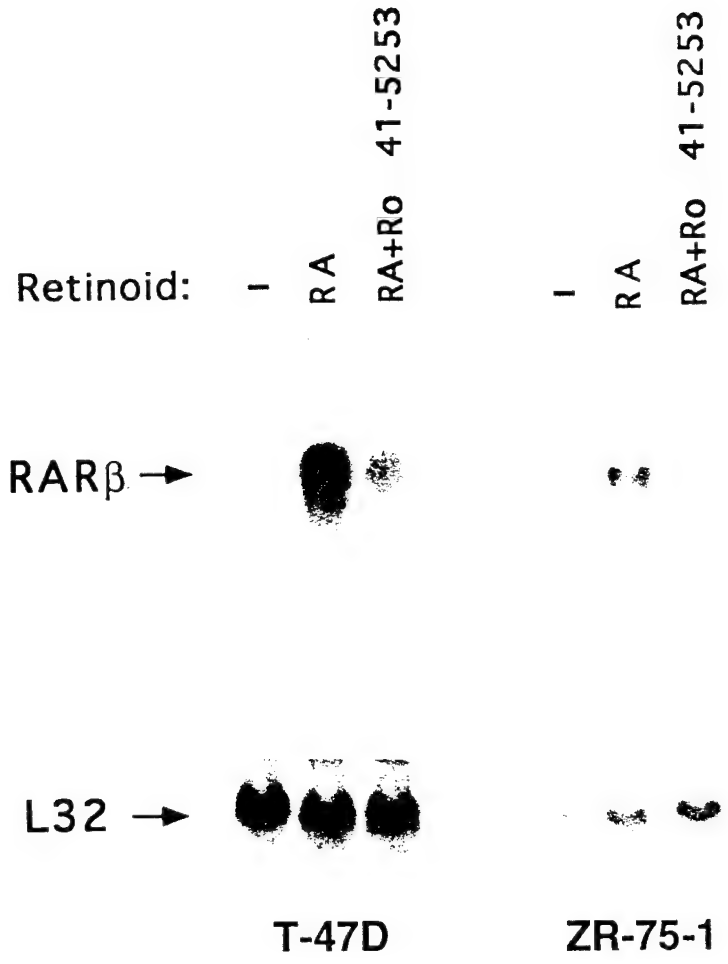

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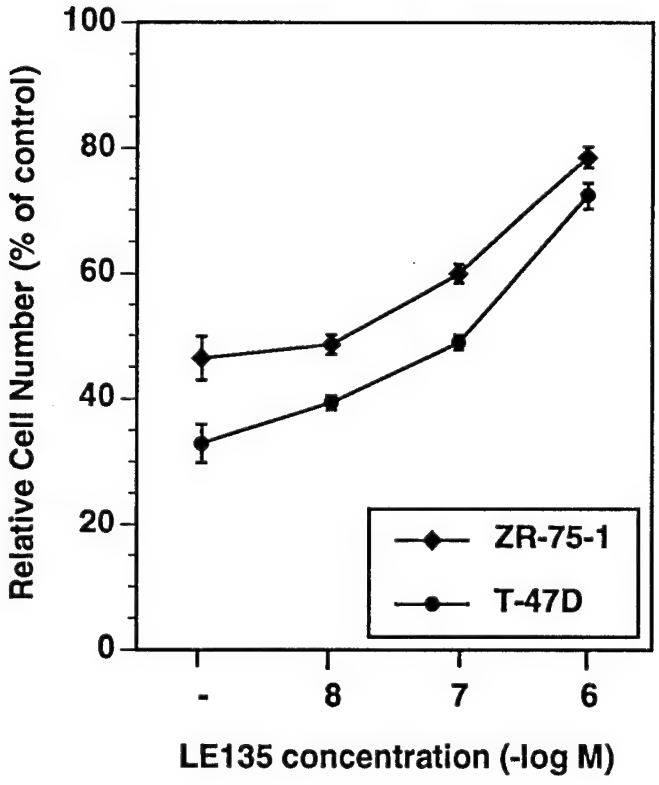

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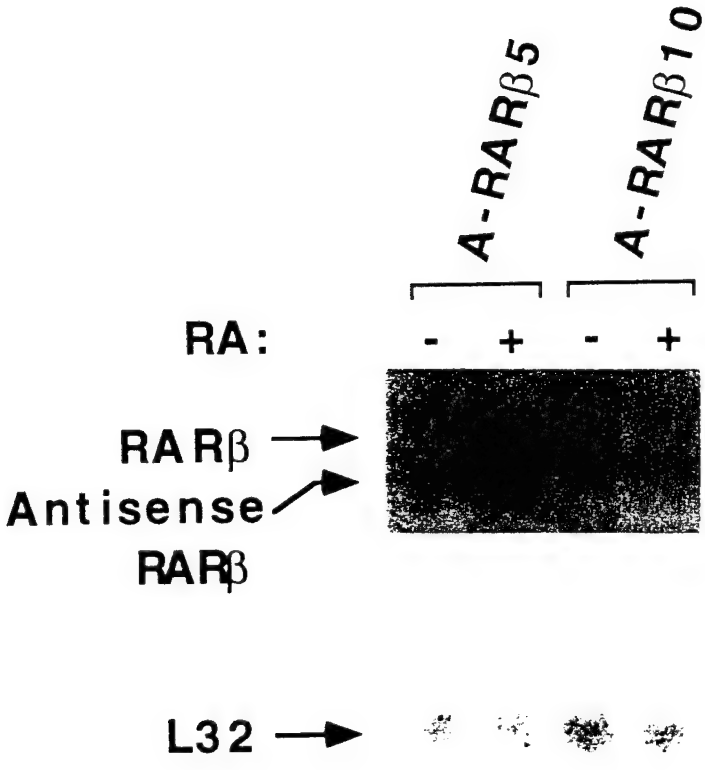

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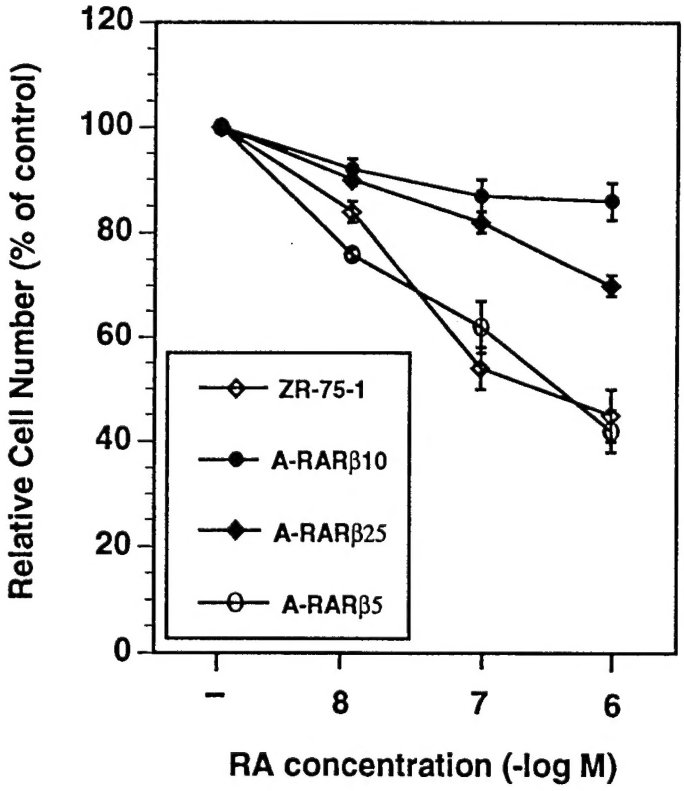

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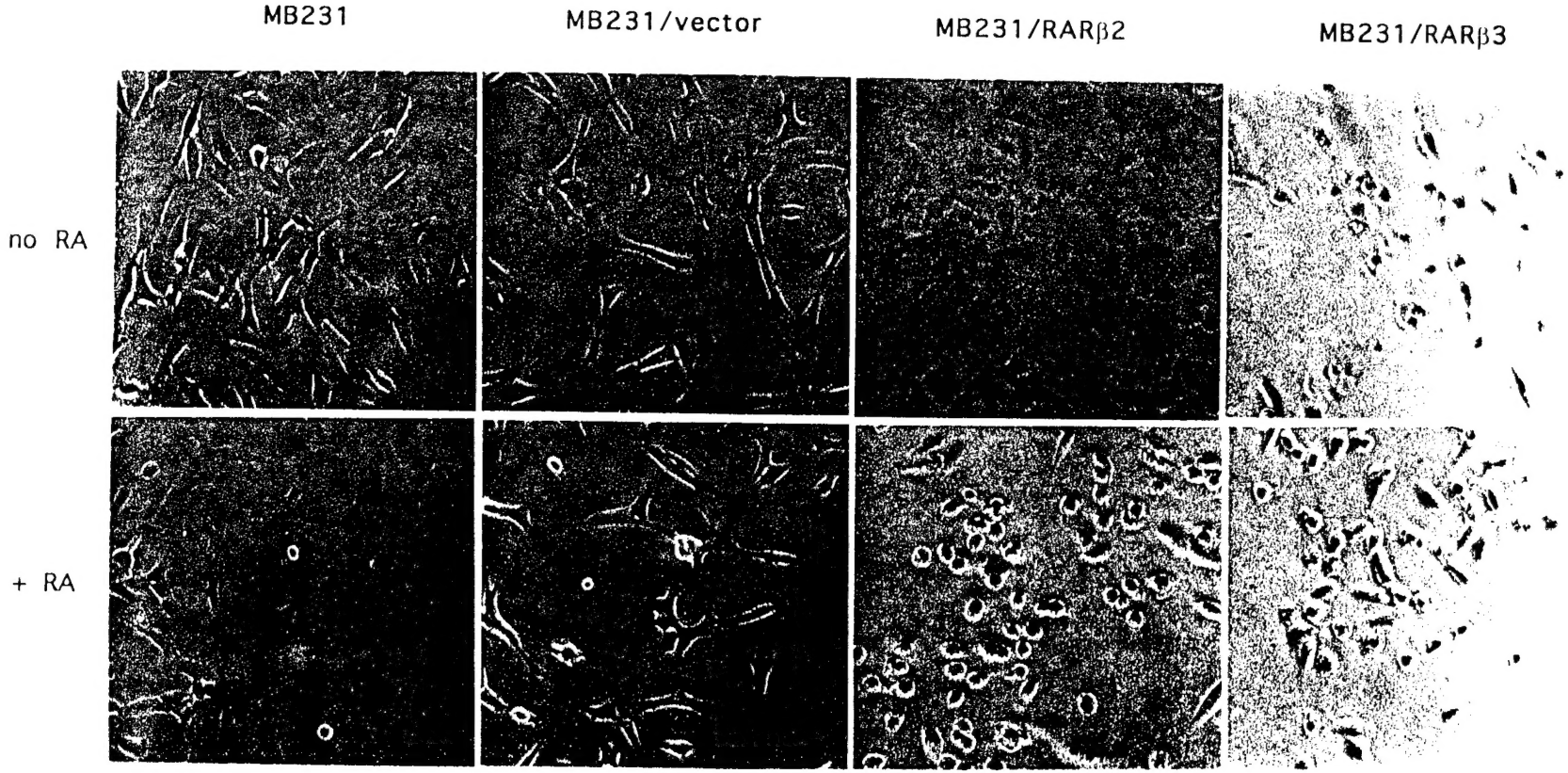

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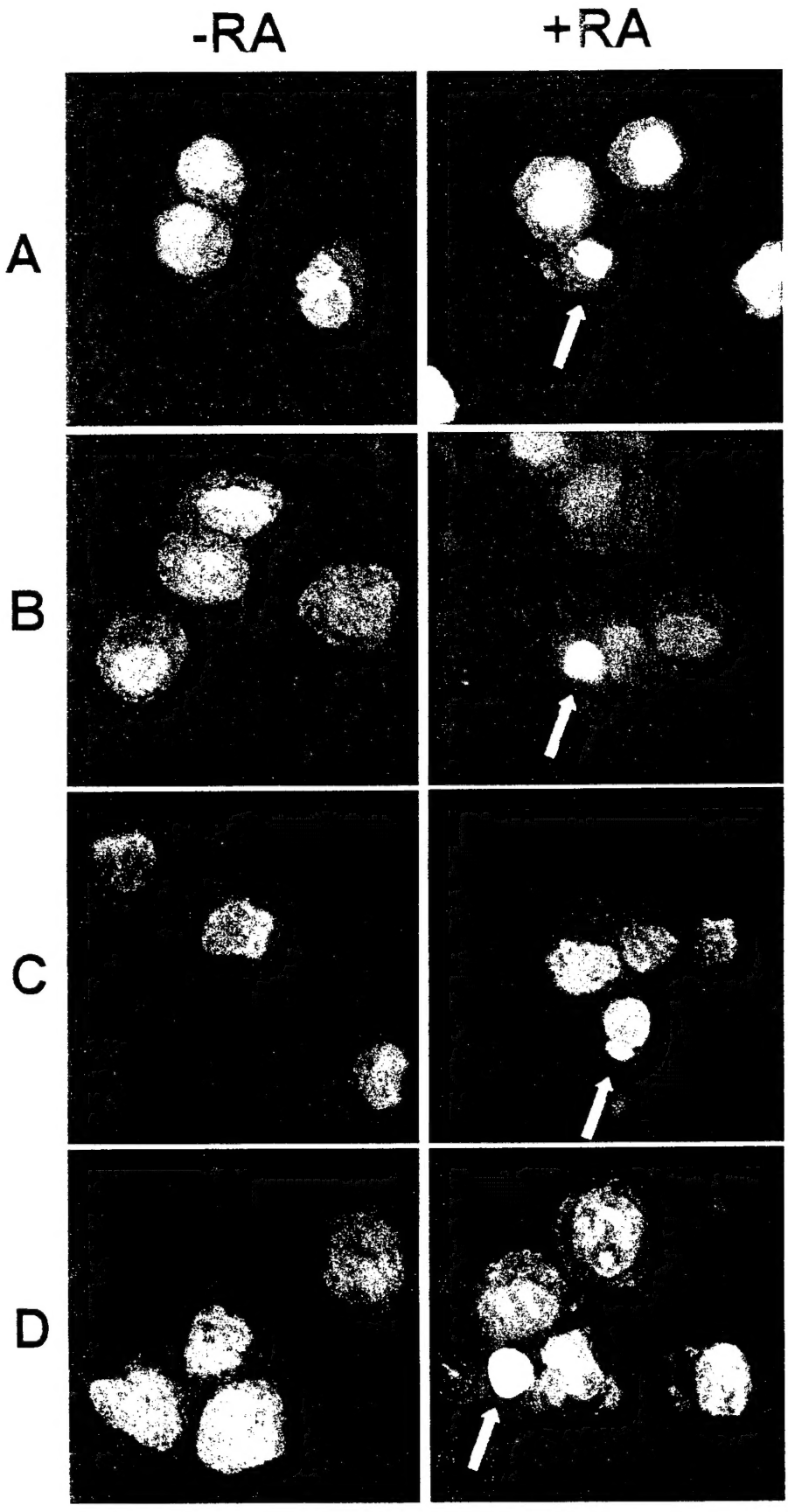

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